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Structure and Function of Gas Vacuoles

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INTRODUCTION

Although many types of cells grow in direct contact with the atmosphere and may depend on the air for their supply of carbon, oxygen, and nitrogen, gas-filled spaces are rarely found inside the protoplasm of living cells. There are, however, certain prokaryotic organisms which possess gas-filled structures of a more or less permanent nature. These structures are known as gas vacuoles. They were first reported towards the end of the last century as occurring in a few species of bacteria (50) and blue-green algae (22). Their bright, refractile qualities and reddish appearance under the light microscope seemed to distinguish them from other granules which had been observed in these organisms, though for some time the nature of their contents remained a matter of dispute, and a variety of synonyms [Höhlungen (117) or hollow bodies, aerosomes (67), pseudovacuoles (59, 63), and Schwebekörperchen (67) or flotation bodies] were used to describe them. Evidence supporting the idea that they contained gas was first provided in 1895 by three German microbiologists, Ahlborn (1), Klebahn (45), and Strodtmann (91), who showed, in the now classical "Hammer, Cork and Bottle" experiment, that gas vacuoles could be made to disappear on the application of a moderate pressure. When a stout bottle was filled to the brim with a suspension of gas-vacuolate algae, a cork applied and struck sharply with a hammer, the pressure generated on the suspension caused the immediate disappearance of the structures. This was indicated by the sudden decrease in turbidity of the suspension, and confirmed by subsequent microscope examination. If the alga had initially been in a floating condition, as was usually the case when gas vacuoles were

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abundantly present in the cells, then loss of buoyancy invariably accompanied the disappearance (see Fig. 1 and 13).

In a series of more detailed experiments that he carried out some years later with highly concentrated suspensions of alga gathered from waterblooms, Klebahn (46–48) demonstrated that the application of pressure resulted in a permanent volume decrease which he took to be equal to the volume of vacuoles which had been destroyed. By comparing this change with the concomitant increase in specific gravity of the algal material, he concluded that the content of the gas vacuoles must have been of very low density, similar to that of a gas, and he pointed out that the compressibility of the structures also indicated gaseous contents. Analyses which Klebahn performed on the gas he collected from gas-vacuolate algal suspensions suggested to him that the gas was probably nitrogen. However, while the results of his experiments are substantiated by recent investigations into the nature and origin of the vacuole gas (105), they now require fresh interpretation (*see below*).

It was apparent to Klebahn that gas vacuoles differed from simple gas bubbles in several respects. Firstly, he observed that they were rarely spherical and usually had an irregular outline. Secondly, the pressure of the gas they contained was, he estimated, substantially less than would exist in bubbles of comparable size. And thirdly, they persisted indefinitely in material placed under a vacuum for prolonged periods of time. For these reasons, he proposed that the vacuole gas must be enclosed in a membrane (46) whose rigidity provided protection from the effects of surface tension and a certain degree of externally applied pressure (a concept which still stands), and whose impermeability prevented the gas from diffusing away (an idea which is no longer supported, *see below*).

Further details of Klebahn's experiments, and of other contemporary physiological studies on gas vacuoles in blue-green algae, can be found in the review made by Fogg (22) in 1941. With the many uncertainties left by these studies, he was unable to reach any firm conclusion as to the functional significance of gas vacuoles in these organisms. This present review takes up the story where he left it and attempts to see whether the information that has since been acquired on the structure, composition, physical properties, and formation of gas vacuoles in blue-green algae and bacteria provides support for any of the functions with which these curious structures have been attributed.

GAS VESICLES

Klebahn's suspicions on the existence of a membrane were confirmed in a rather unexpected way by electron microscope studies that were carried out many years later. In 1965 Bowen and Jensen (4, 5), using thin-sectioning techniques, found that, in five species of blue-green algae they investigated, the gas vacuoles

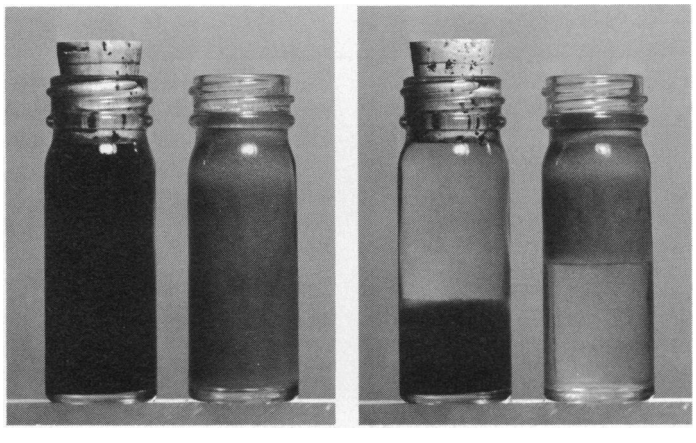

FIG. 1. *The "Hammer, Cork and Bottle" experiment, showing two results of collapsing the gas vacuoles in the blue-green alga Microcystis aeruginosa. (a) The decrease in turbidity after striking the cork in the left-hand bottle (the bottle on the right is untreated). (b) The loss of buoyancy apparent in the treated sample after allowing it to stand for a couple of hours.*

were made up of stacks of cylindrical, electron-transparent structures which they termed "gas vesicles." Hollow, and bounded by single membranes only 2 nm thick, the vesicles were reported to have the form of cylinders, of constant diameter (75 nm) but variable length (200 to 1,000 nm), with conical ends. Bowen and Jensen could not detect these cylindrical structures in algae which had been subjected to a pressure sufficient to destroy their gas vacuoles, and they correctly concluded that the vesicles had collapsed flat to the double, membranous elements which they found in their place.

Simultaneously, Jost (40), investigating the fine structure of another blue-green alga, *Oscillatoria rubescens*, by the newly-developed freeze-etching technique, produced very fine pictures of similar structures which, presuming they were filled with water, he called "Hohlspindeln" (hollow spindles). They were subsequently recognized to be identical with gas vesicles, however (44). The gas vacuoles of thirteen species of blue-green algae from eight genera have now been studied by electron microscopy (3, 4, 40, 44, 66, 80, 84, 96, 101, 114), and in each case gas vesicles of the same basic pattern have been found (see Table 1). The structures have been variously termed "gas vacuoles" (101), "gas cylinders" (85, 112), and "gas vacuole subunits" (105), but the original term gas vesicle is used in this review, as it is unambiguous and makes no assumptions about shape when comparing with homologous structures from other groups of organisms.

Although it is in the blue-green algae that gas vesicles have recently attracted most attention, they were first seen by electron microscopy in the extremely halophilic bacterium *Halobacterium halobium* by Houwink in 1956 (34). His studies showed the gas vacuoles to be made up of hollow structures, much smaller and more numerous than the optical microscope revealed. In the absence of a satisfactorily developed thin-sectioning technique, stereo-pairs were used to demonstrate the three-dimensional structure of the vesicles, which plainly survived without collapse in the desiccated bacteria. The vesicles were reported to be lens-shaped, though this form was reckoned to have resulted from distortion of an initially spherical morphology. Apart from these original contributions, Houwink also correctly interpreted the collapse of gas vesicles by the pressure developed on centrifugation. Regrettably, Houwink's ingenious work and sound observations remained rather neglected, perhaps because the techniques available to him did not permit him to demonstrate the presence of the enclosing membranes. Indeed, this was not done until 1967, when Larsen, Omang, and Steensland (57) revealed the existence of the membranes by thin-sectioning and commented on their fundamental similarity to the gas vesicles of blue-green algae. At the same time a much more extensive investigation was reported by Stoeckenius and Rowen (90) describing many facets of gas vesicles of *Halobacterium halobium*, as shown by thin-sectioning, negative staining, and metal shadowing. Unfortunately all of their work was performed on material which had been centrifuged, with the result that the vesicles were seen only in the collapsed state. Stoeckenius and Rowen referred to them as "intracytoplasmic membranes." They recognized that they were "related to the formation of gas vacuoles" in the halobacteria cells, and that they had features in common with the structures characterized as gas vesicles in blue-green algae (4), but it was not until a later publication (89) that their true identity was conceded.

Gas vesicles of homologous form have now been demonstrated in several other bacterial groups, including some of those in which gas vacuoles had been reported by earlier workers on the basis of light microscope observations. A comprehensive list is given in Table 1. Attention is drawn to the possibility that in certain of the forms described in the earlier literature, other refractile granules may have been mistaken for gas vacuoles. For example, the criteria of certain optical properties and solubility in various reagents used to identify these structures in species of *Thiothrix* (117) *Rhodocapsa* (67), *Peloploca* and *Pelonema* (58, 59) are really inadequate. It is worth noting that the occurrence of the structures in each of the last three examples was originally reported by Molisch and Lauterborn who were sceptics of Klebahn's "gas vacuole theory" and that *Rhodocapsa suspensa* (67) is now incorporated, via *Rhabdomonas gracilis* (see *Bergey's Manual*, 7th ed.), in the genus *Chromatium*, which is said to be without gas vacuoles [N. Pfennig and H. G. Trüper, in *Bergey's Manual*, 8th ed., *in preparation*]. There are, however, in certain of the reports from this era (e.g., 50, 70, 71), descriptions of the disappearance under pressure of the structures in question. This behavior is diagnostic only of gas vacuoles in prokaryotic organisms and can therefore be regarded as providing unambiguous identification of these structures where they occur.

TABLE 1. *Prokaryotic organisms in which gas vacuoles have been reported*

Organism[a]	Shape and width (in nm) of gas vesicles, if reported[b]	References
Schizophyceae Cohn		
Chroococcales Wettstein		
Chroococcaceae Nägeli		
Microcystis aeruginosa Kützing emend: Elenkin	cyl. w. 71	41
Coelosphaerium sp. Nägeli		77
Nostocales Geitler		
Oscillatoriaceae Kirchner		
Spirulina platensis (Gom.) Geitler	cyl.	31, 66
Oscillatoria rubescens D.C.	cyl. w. 65	40
O. princeps Vaucher	cyl.	80
O. agardhii Gomont	cyl. w. 70	84
O. redekei Van Goor	cyl.	114
Trichodesmium erythraeum Ehrenberg	cyl. w. 75–85	96
Phormidium sp. Kützing		10, 14
Lyngbya sp. Agardh		59
Nostocaceae Kützing		
Anabaena flos-aquae (Lyngb.) Breb	cyl. w. 70, 75	84, 108
A. spiroides Klebahn	cyl. w. 75	4
Anabaenopsis sp. Miller		31
Aphanizomenon flos-aquae (Linn.) Ralfs	cyl. w. 75	4
Nostoc muscorum Agardh	cyl.	100
N. sphaericum Vaucher	cyl.	3
Scytonemetaceae Rabenhorst		
Tolypothrix sp. Kützing		10
Rivulariaceae Rabenshorst		
Calothrix sp. Agardh.		10
Gloetrichia echinulata (J. E. Smith) P. Richter	cyl. w. 70	84
Schizomycetes Nägeli		
Pseudomonadales Orla-Jensen		
Thiorhodaceae Molisch		
Lamprocystis roseopersicina (Kützing) Schroeter		*Bergey's Manual*, 8th ed., *in preparation*
Thiodictyon sp. Winogradsky		*Bergey's Manual*, 8th ed., *in preparation*
Thiopedia rosea Winogradsky	spin., short cyl. w. <120[d]	78
Amoebobacter roseus Winogradsky (= *Rhodothece conspicua*)	spin., short cyl. w. 80–90, <120	13 108
(*Rhodocapsa suspensa* = *Rhabdomonas gracilis* = *Chromatium* sp.)[c]		67
Chlorobiaceae Trüper and Pfennig		
Pelodictyon clathratiforme (Szafer) Lauterborn	cyl. w.75	73
Clathrochloris sulfurica Geitler		*Bergey's Manual*, 8th ed., *in preparation*
Pseudomonadaceae Winslow et al.		
Halobacterium halobium Petter	mostly spin., w. <250[d]; cyl. w. <180[d]	89
H. strain Delft	mostly spin., w. <250; cyl. w. <137	13
H. strain 5	mostly spin., w. <300, <198	57 108

[a] Bacteria (*Schizomycetes*) classified as in *Bergey's Manual of Determinative Bacteriology*, 7th ed., except for *Thiorhodaceae* and *Chlorobiaceae*, classified according to Pfennig and Trüper (*Bergey's Manual*, 8th ed., *in preparation*). Blue-green algae (*Schizophyceae*) classified according to Desikachary (14).

[b] spin. = spindle shaped; cyl. = cylindrical; w. = width; < denotes largest dimension recorded.

[c] Criteria for gas vacuoles doubtful and require confirmation (see text).

[d] Estimated from micrographs in publication.

TABLE 1—*Continued*

Organism[a]	Shape and width (in nm) of gas vesicles, if reported[b]	References
Spirillaceae Migula		
Microcyclus aquaticus Orskov	cyl. w. 68–125	97
Chlamydobacteriales Buchanan		
Peloplocaceae Beger		
Peloploca sp. Lauterborn[c]		58
Pelonema sp. Lauterborn[c]		51, 59
Eubacteriales Buchanan		
Achromobacteriaceae Breed		
?*Achromobacter* sp. Bergey et al.		98
Micrococcaceae Pribram		
Sarcina ventricula Goodsir emend. Beijerinck		33, 50
Beggiatoales Buchanan		
Beggiatoaceae Migula		
Thiothrix tenuis Winogradsky[c]		115
Classification as yet undecided		
Prosthecomicrobium pneumaticum Staley	short cyl., spin. w. <200[d]	87
Ancalomicrobium adetum Staley	short cyl., spin. w. <200[d]	

STRUCTURE OF THE MEMBRANE

Support for the idea that the gas vesicles in these widely separated groups of prokaryotic organisms are homologous structures comes from the striking similarity of the membrane in each case. It is markedly thinner than the typical cell unit membranes, being only 2 nm wide; it has a characteristic striated appearance, being banded by ribs 4.5 nm wide running at right angles to the long axis of the structure; and the ribs seem to be made up of particles. The evidence for this, presented in detail below, is drawn from published accounts of electron microscope studies, and from X-ray diffraction studies carried out by A. E. Blaurock, W. Wober and W. Stoeckenius (at San Francisco Medical Center) on the gas vesicles of *Halobacterium halobium*, and subsequently by A. E. Blaurock and myself (at London University) on those of *Anabaena flos-aquae*.

Membrane Thickness

In thin-sectioned material the membranes in all the organisms investigated have shown good preservation in osmic acid (4, 13, 84) but not in permanganate (84) unless post-stained in uranyl acetate (90) which itself proves a good initial stain (57). Prefixation in glutaraldehyde (13) or formaldehyde (90) gives good results and is essential in the case of the halobacteria (13, 57) which otherwise show immediate lysis. If the gas vesicles remain intact during preparation, and are cut normal to either the major or minor axes, the stained membrane appears as a single line, or perhaps as a series of touching dots, of mean thickness given variously as 2 nm (4, 73, 84), 2 to 3 nm (13), or 3 nm (57, 84). Because of the danger of gas vesicles collapsing flat on centrifuging, several workers have preferred to use membrane filtration as a means of concentrating material during the fixing and staining procedures (13, 57, 89). Centrifugation may be used however, provided low accelerations are used (*see below*). The material may concentrate at the surface during the initial stages of preparation, due to the buoyancy imparted to it by the gas vesicles, though it invariably sinks after absorption of heavy stain.

In section, collapsed membranes appear as three-layered structures, the narrow central region of low electron density indicating either that the two adpressed inner surfaces contact one another over a relatively small proportion of their area, or that the stain does not penetrate completely. The half-thickness of the collapsed structure should give a better estimate of the membrane's mean width. The total thickness of the collapsed structure has been given as 8.0 nm in the halobacteria (90), 6.0 nm in the blue-green algae (4) and 5.0 to 5.5 nm in the photosynthetic bacteria (13), though all of these values should be considered as being only approximate. Jost and Jones (41) point out that the dimension may be affected by staining and report a value of 2.8 ± 0.4 nm for the thickness of the *Microcystis* gas vesicle membrane, from measuring the lengths of

shadows cast by vesicles collapsed flat on Formvar-coated grids. Even with this method errors may arise due to uncertainties in the shape of the profile at the edge of the collapsed vesicle, and to the difficulty of estimating the thickness of shadowing material deposited on the specimen itself, and it is emphasized that these estimates are also challenged by the resolution of the technique.

The best estimate of the wall thickness comes from X-ray diffraction studies. The first of these was made with the gas vesicle membranes of *H. halobium* by A. E. Blaurock and his collaborators, who produced a layered structure by drying an aqueous dispersion of collapsed vesicles onto a smooth surface and analyzed the X-ray reflections recorded on photographic film. The X-ray beam was tangent to the surface, and the photographic film was placed perpendicular to the beam a few centimeters from the dried dispersion. The diffraction pattern obtained indicated a series of sheets layered at a repeating interval of 3.8 nm. By comparison with the pattern obtained from an aqueous dispersion of collapsed vesicles, it was concluded that this measurement represented the thickness through the two opposed walls of the collapsed vesicle and that the mean thickness of the vesicle wall is therefore 1.9 nm. However, metal-shadowed replicas of freeze-fractured (40, 101) and isolated (41) gas vesicles give evidence that both the inner and outer surfaces are corrugated (see also Fig. 12a). Consequently, the overall thickness may be greater than 1.9 nm if the corrugations of opposing surfaces interdigitate, as indicated in Fig. 2a. Studies in progress on gas vesicles isolated from *Anabaena flos-aquae* have shown that the molecular structure is similar to that of the *H. halobium* vesicles, no significant difference having yet been found in corresponding X-ray diffraction patterns.

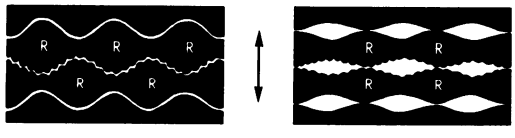

FIG. 2. *Diagram showing two ways in which the corrugated surfaces of successive layers of collapsed gas vesicles might, in theory, pack together; (a) interdigitating, and (b) not interdigitating. Arrow represents the repeating interval (3.8 nm) that would be generated in both cases. R denotes the ribs in cross section. Wavy edge represents the inner, and smooth edge the outer surface of the collapsed vesicle.*

Rib Spacing

Jost (40) was first to show by freeze-fracturing that the inner surface of the membrane was clearly striated, ribs running round the structure approximately at right angles to its long axis and at a period given as 4 nm. It is always the inner surface which is exposed by fracture of frozen specimens, but the outer surface may be exposed by subliming away the encasing ice, and this reveals a similar ribbed topography (41, 99). The ribs show with less contrast on the outside, perhaps because of contamination by nonvolatile material left after etching, but then the contrast is also low in metal-shadowed preparations of isolated vesicles (D. Branton, *unpublished data*; see figures in reference 41). More contrast is revealed by uranyl acetate (13) and phosphotungstate (112) negative stain, which presumably lodges in troughs between the ribs on the outer surface. The rib period appears somewhat larger, 5 nm (86, 90, 112) on staining, however. The presence of 4-nm (13, 57) or 5-nm (90) wide ribs is also seen in sections parallel to the long axis of the vesicle. Recently, we have attempted to obtain a more accurate estimate of the rib spacing by analyzing the electron microscope images of gas vesicles, isolated from *Anabaena flos-aquae*, by the optical diffraction methods described by Klug and Berger (49), and we have obtained values of 4.5 nm from freeze-fractured material, 4.9 nm from vesicles metal-shadowed after drying flat on Formvar-coated grids, and between 4.8 and 4.9 nm with various negative stains. The value from freeze-fracturing would seem to be the most dependable as the other methods appear to be accompanied by distortion during drying and staining. Moreover, the value 4.5 nm is the same as that obtained by direct X-ray diffraction analysis of the untreated intact vesicles of both *Halobacterium halobium* and *Anabaena flos-aquae*.

Stoeckenius and Kunau (89) have pointed out that the ribs indicate a structure comprising either a stack of hoops or "one or several continuous strands wound into a conical helix of low pitch," in the halobacterium membrane (and by extension, into a cylindrical helix in the central region of the blue-green algal type of gas vesicle). Observing short striae resembling dissociated ribs in old preparations of disintegrated gas vesicles, they favored the hoop model. Jost and Jones (41) on the other hand, observing that some ribs liberated from algal vesicles exceeded in length the circumference of the vesicle, preferred the helix model, and they claimed to be able to

detect the calculated angle of inclination (2.5°) of the ribs to the long axis, from stereoscopic micrographs, though the evidence for this has not yet been formally presented. It might be thought that the controversy could be settled by analyzing the images of negatively stained collapsed membranes. Assuming that the structures were stained on both sides, and that apart from being collapsed flat they were not otherwise distorted, hooped and spiralled cylinders when flattened would be expected to give the sort of images depicted in Fig. 3a and 3b, respectively. Even though it might not be possible to resolve the two sets of ribs in case (b) due to the narrow angle θ (= 2.5°) and small rib period (4.5 nm), (a) and (b) should be readily distinguishable because in (a) the intensity of the banding would be the same right across the width of the structure (as is observed, see Fig. 12c) whereas in (b) the contrast would be greater at the edges where the ribs at the back and front overlap. Superficially then, the homogeneous appearance of the collapsed structure would seem to lend support to the hoop model. However, these considerations presuppose that the basic hooped or spiralled format is retained on collapse, and in fact this may not happen. The ribs must anyway be broken along the edges of the collapsed structure, and this would give the two halves of each rib the freedom to reposition themselves. For example, were the ribs to be turns of a spiral, on collapse those in one half of the membrane might come to lie opposite those in the other half (as in Fig. 3a) or in the troughs between them (as in Fig. 3c) where they would give closer packing. X-ray diffraction studies indicate that the latter occurs, as the first-order reflection generated by the 4.5-nm rib period disappears on collapsing the membrane.

We are thus unable to settle the simple matter of how the ribs are wound in the structure at present. The unravelling of this problem is, of course, essential to the solution of the gas vesicle structure and to an understanding of gas vesicle assembly.

Subunit Structure

Micrographs of freeze-etched gas vesicles suggested to some that the ribs might be made up of globular subunits (40, 41, 100), and the same has been claimed from their negatively stained appearance (112), though in the view of Stoeckenius and Kunau (89) the evidence is unconvincing. Nevertheless, Jost and Jones (41) have pursued this idea, claiming that particles occur at intervals of between 2.8 and 3.5 nm. From these measurements and their measurements of membrane thickness (2.8 ± 0.4 nm) and rib period (4.0 ± 0.2 nm), they propose that the gas vesicle membrane of the blue-green alga *Microcystis aeruginosa* is made up of ellipsoid particles whose three principal axes measure 3.0, 2.8 and 4.0 nm, respectively. Now, if the major axis is only as large as the rib period (4.0 nm), then it must be assumed that the ellipsoids in one rib are aligned with those in the two adjacent ribs, as indicated in Fig. 4a. If the particles are ellipsoids, there is no reason why they should not interdigitate, as shown in Fig. 4b. With this hexagonal stacking arrangement, the length of each ellipsoid would be $2/\sqrt{3}$ times larger than the rib period (giving a major axis of 4.6 nm).

The model proposed by Jost and Jones may seem plausible at first sight, but it is open to certain objections (*see below*). The dimensions under consideration are not much in excess of what is generally reckoned to be the limit of

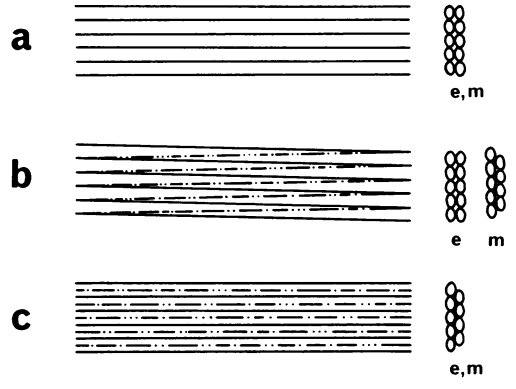

FIG. 3. *Diagram showing three ways in which, theoretically, the ribs might be arranged in the collapsed vesicle; face views and profiles, e at edge, m at middle. Solid lines denote ribs at front; broken lines at back; the two coincidental in (a).*

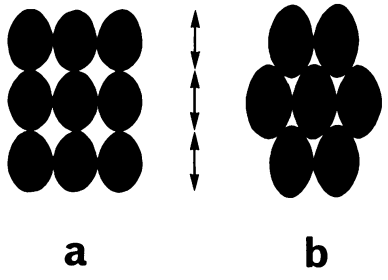

FIG. 4. *Diagram showing two ways in which hypothetical ellipsoid particles might be stacked, (a) rectangularly, and (b) hexagonally. The regular "rib" period, indicated by the arrows, is the same in both cases, even though the particles in (b) are larger.*

resolution of biological structures by electron microscopy (about 2 nm) due to the granularity imposed by staining and metal-shadowing techniques. Even the smooth Formvar support may look granular when slightly underfocused (as in Fig. 12b), and this could lead to misinterpretation when superimposed on linear structures like the ribs. The images of the membranes need detailed analysis by the sort of averaging techniques which have been used in determining the substructures of certain viruses (e.g., 49, 65). As yet our optical diffraction studies on the images of shadowed, stained, and freeze-fractured gas vesicles from *Anabaena flos-aquae* (D. Branton and A. E. Walsby, *unpublished data*) have failed to reveal any repeating subunit in the plane of ribs. And a second cautionary note should be sounded here. Certain authors (13, 57, 86) have proposed a globular or beaded substructure on the basis of the dotted appearance of the membranes in thin section. However, this appearance is *only* given when the vesicle is cut across the ribs (as described above). Of course, the lack of a dotted appearance in sections running in the plane of the rib does not, by itself, prove that the ribs are *not* composed of globular subunits. Globules of the dimensions under consideration (~3 nm) would only be detected in 30-nm "thin" sections if they happened to be arranged in ranks perpendicular to the plane of the section. It seems to invalidate the model portrayed in Fig. 4a, however, as this would give rise to just such a ranked arrangement.

Recently, Blaurock and I have prepared samples of partially orientated gas vesicles which have permitted X-ray studies of the subunit structure of the ribs. These samples have been obtained by drying drops of a highly concentrated, aqueous suspension of intact *Anabaena flos-aquae* vesicles between two glass points, the vesicles apparently tending to align side by side as a consequence of having a high axial ratio. According to Blaurock, the diffraction patterns obtained indicate that there are subunits packed in a crystalline fashion, but it is not clear whether they have any correspondence with the subunits suggested by the electron microscope studies. The interpretation of these results is summarized in a later section as it presupposes knowledge of the chemical composition of the gas vesicle membrane.

CHEMISTRY OF THE MEMBRANE

The investigations of early workers (46, 67), reviewed in detail by Fogg (22), did not give any exact information on the chemical composition of gas vacuole membranes, and in fact Klebahn's suggestion that the disappearance of gas vacuoles in the presence of various hydrocarbons indicated a lipoid structure (46) has since proved misleading. The unusual staining properties of gas vesicles prepared for electron microscopy, while indicating a chemistry different from that of typical unit membranes (4, 84), again provided no positive information; this had to await the development of techniques for isolating and purifying these structures.

Methods of Isolation

Jost and Matile correctly reasoned that, like gas vacuoles, the constituent gas vesicles should have a specific gravity substantially less than that of water, and that consequently it should be possible to separate them from other cell components by centrifugally accelerated flotation (43). They reported the isolation of gas vesicles from the blue-green alga *Oscillatoria rubescens* by two-stage centrifugation of a cell brie obtained by grinding the alga with glass beads. However, the procedure they employed has been questioned on the grounds that (i) the structures they isolated did not possess the characteristic shape and fine structure (86, 89) which gas vesicles have been shown to retain on isolation (112); (ii) the fraction they obtained was of a very different composition from the well-characterized preparations subsequently obtained (38, 89, 111), in having lipophilic rather than proteinaceous components; (iii) gas vesicles would not have survived the pressures generated by the high centrifugation speeds they employed (89). Intact gas vacuoles have now been obtained from blue-green algae and halobacteria by several groups, using various methods which ensure that these sensitive structures are not subjected to damaging pressures during cell disruption and the subsequent purification procedures.

Cell lysis. The problem of breaking open gas-vacuolate cells without subjecting gas vacuoles to pressure was first solved in principle by Petter (71) who found that the bodies were released intact when *Halobacterium halobium* was killed on diluting the culture medium with water. Stoeckenius and Kunau (89) employed a modification of this method, gradually diluting the medium by dialyzing the culture against distilled water. Larsen et al. (57) found that the cells of halobacteria would lyse spontaneously on elevating the *p*H of the culture medium by adding sodium hydroxide. Neither of

these methods are effective with other gas-vacuolate bacteria or blue-green algae, but we have found that algae of *Anabaena* and *Nostoc* type lyse rapidly in strong solutions of sucrose (0.7 M) which cause osmotic shrinkage of the protoplasts (111). This method does not work well with the more robust unicellular forms, though these can be broken by osmotic shock after impregnation with glycerol, as long as the cell walls have been weakened by incubation in penicillin (200 units per liter) in the presence of the magnesium ion (38). Some other blue-green algae lyse much more readily (13), allowing gas vesicles to be collected from the autolysates of old cultures.

Centrifugation. Both Larsen et al. (57) and Stoeckenius and Kunau (89) found that gas vesicles could be collected by centrifugally accelerated flotation, but the difficulties were such that they preferred to use membrane filtration (of intact vesicles, 57) and density gradient ultracentrifugation (of collapsed ones, 89) in their purification, though it appears that neither method will by itself yield preparations of very high purity (38). However, we have found (9, 111) that it is possible to obtain centrifugation conditions which give good yields (50% recovery) of gas vesicles in a high degree of purity (97.5%). This depends on first determining the maximum pressure that can be applied to the gas-vacuolate suspension without causing any collapse (about 2.5 atm with blue-green algae, but only 0.4 atm with the halobacteria; see Fig. 9), and then seeing that the pressure developed by centrifugation (equal to $h\rho g$, where h is the depth of suspension, ρ its density, and g the centrifugal acceleration) does not exceed this value (111). By repeatedly centrifuging the gas vesicles to the surface, ideally through distilled water or buffer layered over the suspension, the vesicles are washed free of soluble material and separated from particulate components which either precipitate or do not float up as quickly (9). This technique has been coupled with liquid polymer partitioning (which removes certain contaminants) and a final ultracentrifugation (permitting recovery of intact vesicles from a shallow layer at the surface) (38), but these additional procedures do not seem to be essential to the purification process (9). A more detailed account of the various isolation procedures is to be found elsewhere (110).

Two groups have used radioactive tracing techniques to assess the per cent purity of the gas vesicle preparations given by their isolation procedures (9, 38). This was done by reisolating previously purified, nonlabeled vesicles after mixing them with (supposedly) homogeneously labeled cell lysates of known specific activity which had been subjected to pressure to destroy any (labeled) gas vesicles present (8). The degree of contamination of the final preparation was then calculated from *its* specific activity. This procedure probably gives a fairly good indication of purity, though it would not detect contamination by substances which become adsorbed irreversibly to the gas vesicle surfaces at sites complexed during the initial isolation.

Chemical Analysis

The proteinaceous nature of the halobacterium gas vesicle membrane was first alluded to by Larsen et al. (57); Stoeckenius and Kunau also thought protein to be the major component, the amino acids released by acid hydrolysis accounting for 70% of the total dry weight (89) even without allowing for hydrolysis losses. A small amount of hexosamine was also found to be present, but no lipid was detected. Krantz (52) reports an even higher proportion of protein, 78% by weight, with non-ashable material accounting for much of the residue. This may represent salts of the protein or salt unremoved by his dialysis procedure. He also records that phosphate and galactose account for a small proportion (2% and 1.5%, w/w, respectively) of his preparations though these were of uncertain purity. However, the fact that the phosphate was not separated from the vesicles by isoelectric focussing, and the galactose was not separated by gel filtration, suggested to him that these components may form an integral part of the structure. If this is the case, then these vesicles would seem to differ from the algal ones, where neither phosphorus (38) nor sugar (9, 38) has been detected.

Gas vesicles from blue-green algae have also, on reinvestigation, proved to be highly proteinaceous (8, 9, 38, 111). Careful analyses have shown that no lipids or carbohydrates (the other major components of unit membranes) are present (8, 38). On the basis of this, Jones and Jost (38) have concluded protein is the only component of the membrane. This view is supported by their quantitative amino acid analyses showing protein accounting for 95% of a sample having about 95.5% purity.

Jones and Jost have proceeded to suggest that not only is protein the sole component of the gas vesicle membrane, but also that only one species of protein may be present (38).

They found that the membranes were not soluble in many of the detergent and other systems used to dissolve proteins, but that they were partly soluble in 80% formic acid and phenol-acetic acid-water (2:1:1). The solubilized fraction ran as a single band on polyacrylamide gel electrophoresis at low *p*H; the insoluble fraction which remained at the origin of the gel apparently comprised more of the same protein molecule, as an eluate of it in the same solvent system produced a similar electrophoretogram, with an identical mobile band and, again, a proportion remaining at the origin (36, 39). By comparison with the electrophoretic behavior of other proteins, Jones postulated a molecular weight for the protein of about 14,000, though he points out that this figure may be in error due to differences in charge density (36).

Substantially similar results have been obtained in our laboratory with the gas vesicles of *Anabaena flos-aquae* after solublizing in a phenol-urea-acetic acid-water solvent system (8, see Fig. 5). These results have brought into question a previous investigation by Smith, Peat and Bailey (86) using the same organism. They obtained a preparation containing collapsed gas vesicles (and various other subcellular structures) from the alga and a comparable fraction from another, but non-gas-vacuolate, alga also designated *A. flos-aquae* by Tischer (94), and analyzed them by electrophoresis. They proposed that one prominent protein band present only in the gas-vacuolate sample must have come from the gas vesicles, and suggested it represented a protein subunit of 22,000 molecular weight. However, Buckland has since found that the gas vesicle protein of this alga is insoluble in the solvent system they employed (8), and it is concluded that they must have happened upon a component of some other structure. It seems probable that the molecular weight of *Anabaena* gas vesicle protein is, in fact, of the same order as the *Microcystis* protein. This we have concluded principally from a quantitative amino acid analysis which shows that most of the amino acids occur in integer ratios, suggesting an empirical formula of 143 residues with a total molecular weight in the region of 15,100 (Table 2). The amino acid composition of the *Anabaena* protein is very similar to that of the *Microcystis* protein (38) though it is not possible to make exact estimates of residue differences because with the latter the molar ratios do not yield numbers close to integers in the same way, assuming a single species having a molecular weight in the region of 14,000. This is also the case with the halobacterium gas vesicle protein (89) which though showing an overall similarity to the algal proteins, has markedly more aspartate, a feature of other halobacterium proteins and possibly an adaptation to the saline environment (56, 88), and less leucine. The notable features common to all three membranes are the absence of any sulphur-containing amino acids and the relatively high proportion of residues with hydrophobic side chains, which might determine the postulated hydrophobicity of the inner membrane surface (89, 105) and hydrophobic interactions of the subunits (*see below*).

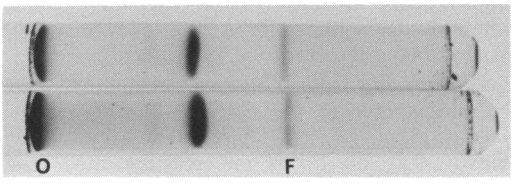

FIG. 5. *Polyacrylamide gel electrophoretogram of the gas vesicles isolated from Anabaena flos-aquae, showing single mobile band. Material staining at front, F, an impurity from the gel, also encountered in unloaded controls. (Reproduced from B. Buckland, reference 8.) The material at the origin, O, is thought to be more of the same protein which has precipitated in the surface layers of the gel [see D. D. Jones (36) and Jones and Jost (39)]. Photograph by courtesy of Barbara Buckland.*

As yet, no estimates of molecular weight have been obtained for the halobacterium gas vesicles. Krantz (52) has remarked that, like the algal gas vesicles, those from *Halobacterium halobium* are not solubilized in solvents which are generally regarded as being suitable for proteins. Hydrochloric acid (12 N), and anhydrous formic acid did produce relatively clear suspensions of collapsed vesicles, but even with these solvents it is doubtful that the membrane was resolved into its individual components, as it was found that the protein could be brought down relatively easily from formic acid suspension by centrifugation.

The successful solubilization of the membrane is, of course, of great importance, not only for the electrophoretic analysis of the protein composition, but also for studies on the production and accumulation of the precursor subunits prior to their assembly into gas vesicles. The difficulties encountered in solubilizing these structures may be connected with the fact that the gas-facing and water-facing

surfaces of the protein molecules have such different properties (*see below*). At the same time, we should entertain the possibility that the protein molecules are held together by covalent cross-linkages.

Relationship of Membrane Structure and Protein Component

Both the thickness of the membrane and the rib period are dimensions which could be spanned by individual protein macromolecules, and it seems likely that the protein component of the membrane indicated by chemical analysis may be arranged as a regular, repeating subunit along the ribs.

The X-ray diffraction studies on the *Halobacterium* gas vesicles by Blaurock and his collaborators have shown that the membrane in cross section consists of two similar parts centered 10 nm apart, and a computed profile shows the peaks of electron density expected for the backbones of two polypeptide chains. In addition there have been observed several prominent wide-angle reflections which characterize an extensive β-protein structure. The wide-angle reflections recorded on the photographic film lie on an axis parallel to the plane of the wall and so confirm the presence of chains running in the wall. Another feature of interest is that the electron density profile of the wall is asymmetric, as predicted assuming that hydrophobic amino acid residues, which are generally less electron-dense than the average, predominate on the inner (gas-facing) surface, whereas hydrophilic residues, which are generally the more electron-dense, predominate on the outer (cytoplasm-facing) surface. These findings correlate well with the results of investigations into the physical properties of the vesicle membrane (*see below*). According to Blaurock, the X-ray study of partially oriented *A. flos-aquae* gas vesicles (*see above*) indicates a repeating "unit cell" from whose dimensions a molecular weight of 7,800 is calculated. Assuming a single species of subunit molecule, the molecular weight is a small multiple of this value, either about 16,000 or 23,000. It appears that some part of the polypeptide chain of each subunit molecule is in the cross-β conformation such that, along any rib, the molecules are joined by hydrogen bonding between the backbones of the chains. There may be two layers of the cross-β structure extending along each rib in such a way as to provide a stiffened structure, which may account for the rigidity of the membrane (*see below*).

TABLE 2. *Amino acid composition of gas-vesicle proteins*

Amino acid	*Anabaena*[a] *flos-aquae*		*Microcystis*[b] *aeruginosa*	*Halobacterium*[c] *halobium*
	Empirical formula	Mole%	(mole%)	(mole%)
Alanine	22.7	15.9	18.6	14.7
Valine	18.0	12.6	11.6	8.8
Glutamic acid	17.7	12.4	11.6	8.6
Isoleucine	14.4	10.1	7.3	9.1
Leucine	14.3	10.0	11.2	0.9
Serine	14.1	9.9	9.3	8.1
Aspartic acid	8.0	5.6	6.4	11.6
Threonine	7.0	4.9	5.2	6.5
Lysine	6.7	4.7	5.4	4.7
Glycine	6.1	4.3	3.4	9.4
Arginine	6.0	4.2	4.9	5.5
Tyrosine	4.0	2.8	3.3	2.3
Proline	2.0	1.4	trace	4.2
Tryptophan	1.0	0.7	1.5	
Phenylalanine	0.9	0.6	0.4	2.5
Histidine	0	0	trace	2.5
Methionine	0	0	0	0
Cysteine	0	0	0	0
(Amide-N)	7.1	5.0		

[a] Analysis by H. Larsen, P. Falkenberg (Trondheim), B. Buckland and A. E. Walsby (Westfield College London).

[b] Data from Jones and Jost (38).

[c] Data from Stoeckenius and Kunau (89). (Table reproduced from B. Buckland, Ph.D. thesis, Univ. of London, 1971.)

The model proposed by these studies departs from the simple globular subunit model (Fig. 4a) in several respects, though it has yet to be verified by an accurate, independent determination of the subunit's molecular weight. In the meantime it is worthwhile reviewing the comparisons that Jones and Jost (38) have drawn between the protein they have separated by electrophoresis and the particles they have postulated from their electron microscope observations. Assuming that each particle is a perfect ellipsoid of volume $4/3\ \pi (2.8 \times 4.2 \times 3.0)/8$ nm (*see above*), they have calculated its molecular weight, by multiplying by the Avogadro number and the density. They estimated the density of the *Microcystis* gas vesicle protein from the amino acid composition and arrived at a value of 1.34 g cm^{-3}. This compares with a value of 1.29 g cm^{-3} determined directly by density gradient centrifugation of the collapsed membranes from this alga (38) [and values of 1.23 g cm^{-3} for the membranes of *Anabaena flos-aquae* (86) and *Halobacterium halobium* (89)]. I would suggest

that the overall density of the collapsed membranes is less than that of the component proteins because a small amount of space is left between the two membrane faces (as shown in Fig. 3), and that consequently the larger estimate, which corresponds more closely with the densities of many other proteins, is probably the better one. Using this figure Jones and Jost arrive at a molecular weight of 14,300. They point out that this coincides with the molecular weight of the gas vesicle protein indicated by its electrophoretic mobility, about 14,000, suggesting that the particles that are seen are the individual protein macromolecules. However, taken at its face value, this comparison demonstrates that, at the outside, there is only just enough protein to permit a grazing, tangential contact between the particles stacked in the least space-filling conformation (Fig. 4a). Such an arrangement is hardly likely to satisfy the requirement the membrane has for structural rigidity (*see below*). This would only be achieved in the context of their model by increasing the area of contact between the particles (e.g., as represented in Fig. 4b); and this, in turn, would require that the particles are smaller in the dimensions through the membrane (as the X-ray studies suggest) or along the rib.

With the idea of corroborating their particulate concept of the vesicle membrane, Jost, Jones, and Weathers (42) have obtained estimates of the average weight of a single gas vesicle. By counting vesicles sprayed in small droplets, whose volume was determined by the addition of a standardized suspension of latex particles, they found that 3.61×10^{11} vesicles of mean length 370 nm had a protein content of 56 μg, i.e., an average vesicle contained 1.55×10^{-10} μg of protein. [Using an entirely different approach we have obtained a similar value for the weight of the *Anabaena flos-aquae* gas vesicle. We measured the total volume occupied by the gas-filled space in a suspension of purified vesicles using a manometric technique (105) and estimated that 31.9 μliters of space was enclosed in 5.3 mg of membrane material (A. E. Walsby and B. Buckland, *unpublished data*). From estimates of the mean dimensions of the average gas vesicle (a cylinder of length 227 nm and diameter 68 nm to the center of the membrane, capped by right cones of altitude 50 nm), we calculate the volume of space in a single vesicle to be 9.45×10^5 nm^3. From this it is estimated that 3.38×10^{13} vesicles were present in the suspension and that the average weight was therefore 1.57×10^{-10} μg. These gas vesicles are, of course, rather shorter than those of *Microcystis aeruginosa*.]

Jost and his collaborators have calculated that the amount of protein they find in a single gas vesicle would be sufficient for 6,700 molecules of 14,000 molecular weight and that this compares with the 6,900 molecules they estimate would be required to cover a right cylinder of length 370 nm, again tacitly assuming the arrangement described in Fig. 4a. If we calculate the area of a *Microcystis* gas vesicle more precisely, taking account of the conical shape of the ends (*see below*), then the value obtained, 7.14×10^4 nm^2, can be covered by about 6,000 of Jost's particles, or by a uniform layer of 1.62 or 1.68 nm (assuming densities of 1.34 and 1.29 g cm^{-3}, respectively). These values are rather less than the figures given by X-ray diffraction, which is to be expected if there are projections on the membrane which prevent complete contact. Similar calculations on the figures for the *Anabaena* vesicle yield a value of about 1.90 nm, the same as the X-ray diffraction value. In both cases the degree of correspondence is encouraging and suggests that it would be worthwhile obtaining more exact estimates of all of the parameters of shape, size, and density used in these calculations.

Implications of the Subunit Model

The general model suggested by these investigations, of a structure built up of just one repeating protein macromolecule, has implications in various fields: firstly, in the processes of gas vesicle formation which may be resolved into those concerned with synthesis of the protein subunits and their assembly into membranes; secondly, in the interactions between the subunits, which determine the overall shape of gas vesicles; and thirdly, in understanding of the physical and chemical properties of the membranes.

Before going on to examine these facets of the gas vesicle in more detail, it is, perhaps, worth digressing to reconsider the description of it as a membrane. The classical unit membrane has two essential ingredients, protein and lipid (and a third, carbohydrate, may also be present). The lipid bilayer has been considered as providing the organizational backbone on which the protein covering is stabilized, and in lacking lipid the gas vesicle would appear to be a very different kind of structure. However, it has been demonstrated that some characteristics of membrane structure remain

after extraction of the lipoid components (21), and the existence of proteinaceous subunits in various types of unit membrane is now being recognized (6). Jones (36) has rightly pointed out that protein-protein interactions will therefore be of general importance and that the gas vesicle may provide an ideal model for studying them.

An alternative view of the gas vesicle has been presented by Smith et al. (83, 86), who draw attention to its similarity to various viral structures which also consist of polymerized protein subunits. In some instances the overproduction of viral coat proteins leads to the formation of empty shells and tubes (65), some of which rather resemble gas vesicles. Smith has made the interesting suggestion that gas vesicles may have originated from viral infections, an attractive hypothesis but one that is difficult to test unless similar processes are continuing in present times. Perhaps when we have gathered more data on the amino acid composition of gas vesicle proteins from different organisms, we may be in a better position to discuss their phylogenetic relationships.

PHYSICAL PROPERTIES OF THE MEMBRANE

The unique feature of the gas vesicle membrane is that it encloses a hollow, gas-filled space, and this poses three questions: how does the gas get inside the membrane; what prevents the membrane from collapsing into the hollow space; and what prevents water from accumulating inside it? Some answers to these questions have been provided by the following studies into the physical properties of the membrane.

Permeability to Gas

The original idea that the vacuole gas must be enclosed in a gas-tight membrane was put forward by Klebahn (46) to meet objections to his "gas vacuole theory" (45) that were made by Molisch (67) on observing that gas vacuoles do not disappear on prolonged evacuation. The idea that the membrane prevented the contained gas from diffusing away persisted until recently, and was extrapolated to the gas vesicles when they were seen by electron microscopy (see 4, 43, 57, 74). However, now that the situation has been investigated it turns out that the gas vesicle membrane is highly permeable to gases (105). The evidence obtained for this is as follows.

(i) By use of a Warburg apparatus which had been modified to enable the gas exchange capacity of a liquid system to be measured, it was found that, when the pressure of the overlying gas phase was reduced, more gas was evolved from a gas-vacuolate suspension than from an equivalent quantity of a suspension in which the gas vacuoles had been collapsed under pressure. When, after equilibrating the suspensions under reduced pressure the system was returned to atmospheric pressure, the gas-vacuolate suspension took up a proportionately greater amount of gas. The extra quantity of gas exchanged in these experiments was equal to the product of the pressure change and the void volume of the gas vacuoles present (as determined independently from the change in specific gravity of the suspension, on collapsing the gas vacuoles). Details of the experiment are summarized in Fig. 6 and 7.

(ii) By use of the same apparatus, it was found that after equilibration of the gas-vacuolate suspension under atmospheric pressure, no pressure change was recorded in the closed system on collapsing the gas vacuoles with an ultrasonic pulse from a transducer located underneath the Warburg flask and then re-equilibrating. Since the gas released was being transferred to an overlying gas phase whose volume was increased by a volume equal to that of the gas vacuoles, the lack of pressure change indicated that the gas had been present in the vacuoles at atmospheric pressure. A parallel experiment indicated that after equilibration under a reduced pressure, the gas pressure in the gas vacuoles had fallen to the same level as that of the overlying gas phase.

(iii) Mass spectrometric analyses showed that after evacuation of a gas-vacuolate suspension to remove dissolved gases, all gases had also been removed from the gas vacuoles present.

(iv) Gas vesicles collapse instantaneously at a well defined range of pressure, when this is applied to them in aqueous suspension as a hydrostatic pressure (108). The required pressure may be generated by the overlying gas phase, but if this is allowed to build up but slowly, the gas dissolves in the surface layers of the suspending medium and diffuses into the gas vesicles there, preventing the differential pressure required to deflate them from being established across the membrane (105). In fact, by using gas vesicles which have been freeze-dried in an intact state (9), it has been found virtually impossible to collapse them in this way, even under rapid rates of gas pressure rise, because the gas leaks inside them so quickly as the pressure builds up.

As yet it has not been possible to give a

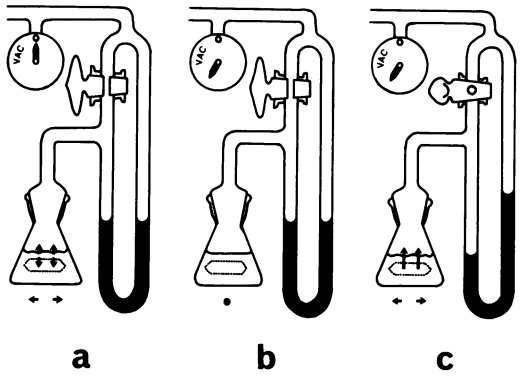

FIG. 6. *Diagram summarizing the use of a modified Warburg apparatus to demonstrate the permeability of gas vesicle membranes to gas, and to determine the volume occupied by the vacuole gas in the sample. (a) Gas-vacuolate suspension equilibrated at atmospheric pressure, Warburg apparatus shaking. (b) Apparatus brought to rest. Pressure of gas phase is reduced rapidly to minimize the loss of gas from the suspension by diffusion. (c) Manometer tap closed. Apparatus set shaking. Gas evolved from the suspension measured by manometry (see reference 105).*

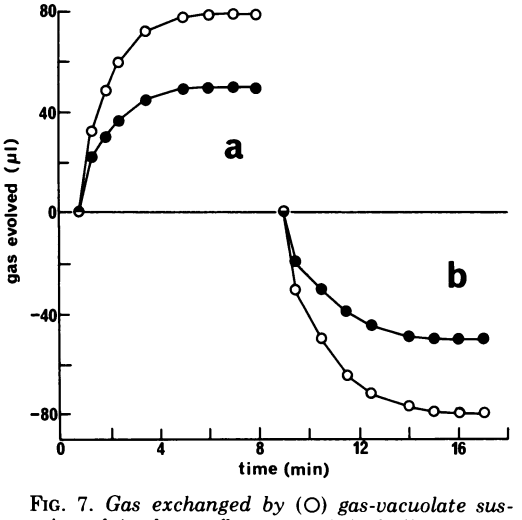

FIG. 7. *Gas exchanged by (○) gas-vacuolate suspension of Anabaena flos-aquae, (●) similar suspension in which gas vacuoles have been collapsed, (a) following reduction in pressure of the overlying gas phase, as described above in Fig. 6, (b) after returning overlying gas phase to atmospheric pressure. Volume of the vacuole gas can be calculated from the difference between the two curves in each case (redrawn from reference 105).*

figure to the gas permeability constant of the membrane, but it is evident that the membrane offers far less resistance to gaseous diffusion than does even a thin film of surrounding water. From the results of manometric and pressure-rise experiments that have been carried out with N_2, O_2, CO_2, CO, H_2, Ar, and CH_4 with gas vesicles of blue-green algae and bacteria (108), it seems likely that the membranes are freely permeable to all types of gases.

Until we know more about the molecular structure of the membrane we can only speculate on the cause of its extreme permeability. However, it is known that the amino acid residues in protein macromolecules, whose structures have been analyzed by X-ray crystallography, can pack together so tightly that very little space is left between them (60, 92). In fact, there may be only a few spaces in a protein macromolecule large enough to accommodate even the smallest gas molecule. It seems very likely that this will also be true of certain regions of the gas vesicle protein. Perhaps the gases diffuse mainly through spaces *between* the protein molecules: indeed *were* the protein subunits perfect ellipsoids stacked rectangularly, and exhibiting only tangential contact (cf. Fig. 4a), the intervening spaces would occupy as much as $(4 - \pi)/4$ (or 21%) of the membrane's surface area.

With the discovery that the membranes are permeable to gases, the identity of the vacuole gas ceases to be even of academic interest! Its composition and pressure will reflect that of the gases dissolved in the surrounding medium, and will normally be air at the ambient pressure, albeit modified by the metabolic activities of the cells. Two important consequences of the permeability are firstly that the vacuoles cannot be used to store gas, and secondly that they cannot be inflated by gas (105).

Rigidity

Arguments for the rigidity of gas vacuole membranes were first put forward in 1922 by Klebahn (46), who observed that the pressure of the gas inside gas vacuoles, only one atmosphere, must be considerably less than pressures exerted on the structures by cell turgor and surface tension. He correctly deduced that the pressure difference must be borne by an enclosing membrane and postulated that the disappearance of gas vacuoles which occurs on application of greater pressures must result either from water being forced into the hollow spaces through cracks in the membrane, or from collapse of the structure. Electron microscopy has shown the latter always to be the case (e.g., 4, 89), the constituent gas vesicles collapsing flat in a plane at right angles to the plane of the ribs (108, 112). Theoretical considerations show that the flattening of the membrane must entail its tearing (108) and, as

this seems to occur preferentially between ribs rather than across them (see micrographs in references 89 and 112 and Fig. 12c), results in the end caps of the cylindrical vesicles splitting off, and spindle-shaped vesicles of the halobacteria splitting at the middle (38, 89). Consideration of the pressures required to collapse gas vesicles leads to the conclusion that they are insufficient to force out the gas as a bubble from the collapsing vesicle, and that it must, rather, escape by diffusion through the membrane (108).

The pressure relationships of gas vesicles inside prokaryotic cells are summarized in Fig. 8. The vesicle collapses when the sum of pressures acting on the outside less the pressure of the gas inside exceeds a certain value, known as the critical pressure. The true critical pressures can best be investigated by using gas vesicles isolated from the cells (so that they are free of turgor pressure) and in a suspension equilibrated with the air (so that g, the pressure in the gas vacuoles, balances a, the atmospheric pressure). Investigations have shown (108) that gas vesicles exhibit a wide, but precisely defined, range of critical pressure, which varies markedly in the different groups of organisms (Fig. 9). In seeking an explanation for this variation, it is worth drawing comparisons with the critical pressures of engineering structures such as pipes and boilers whose critical pressures are determined by the strength of the wall material, their shape and wall thickness relative to overall length, and, particularly, diameter (2). The same factors probably determine the critical pressures of gas vesicles;

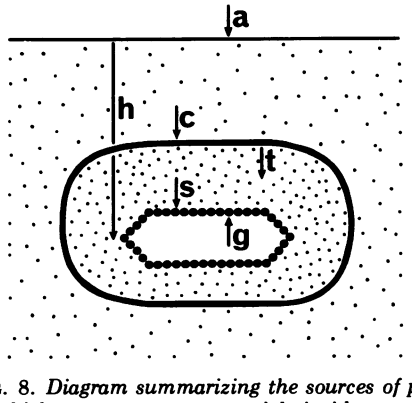

FIG. 8. *Diagram summarizing the sources of pressure which may act on a gas vesicle inside a prokaryotic cell: (a) atmospheric pressure; (h) hydrostatic pressure due to depth; (t) cell turgor pressure; (c) pressure from surface tension at the cell wall; (s) pressure from surface tension at the gas vesicle wall (g) pressure of the vacuole gas. (Reproduced from reference 108 with permission from The Royal Society, London.)*

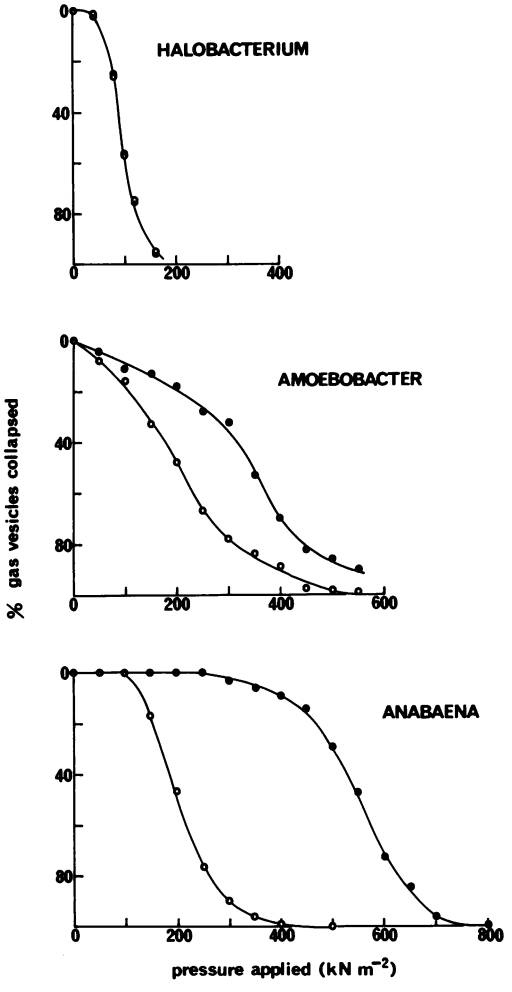

FIG. 9. *Collapse of gas vesicles with pressure in Halobacterium sp. strain 5, Amoebobacter rosea and Anabaena flos-aquae. (O) Cells suspended in culture medium; (●) cells suspended in medium supplemented with sucrose to remove turgor pressure. In each case the turgor pressure of the cells is given by the mean distance between the two curves (redrawn from references 15 and 108).*

it has been found that the gas vesicles of the halobacteria, which tend to be considerably wider than those of the alga (Fig. 10) are also much weaker. The case of the vesicles in a photosynthetic purple bacterium is intermediate between these two (108). However, the inverse correlation between width and strength has yet to be rigorously tested. There seems to be no significant (inverse) correlation between the length and strength of the blue-green algal vesicles (which do not vary in diameter), and this suggests that, unlike cylinders of homogeneous construction, little support is derived

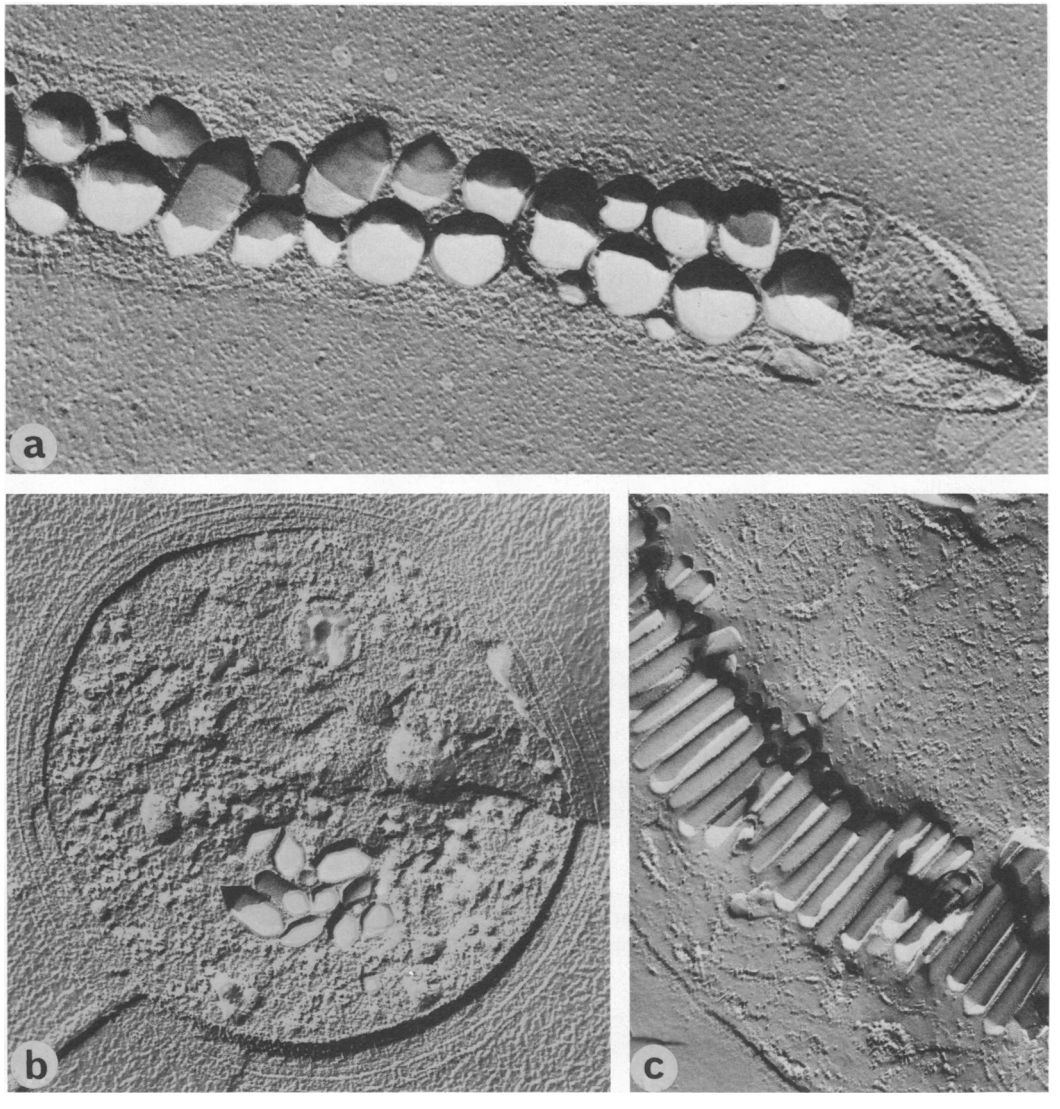

FIG. 10. *Electron micrographs of freeze-fractured cells of* (a) *Halobacterium sp. strain 5,* (b) *Amoebobacter rosea, and* (c) *Anabaena flos-aquae, demonstrating range of size and shape of the gas vesicles, whose inner (gas-facing) membrane faces are exposed. No etching; all* ×*45,000. (Specimens prepared in collaboration with D. Branton and S. Whytock, Univ. of California, Berkeley.)*

from the ends along their length, i.e., the main strength of the structure resides in the individual ribs and there is little lateral support from one rib to the next. The variation in critical pressure of the algal vesicles probably reflects, therefore, variations in the strength of the wall material. Irregularities in assembly of the structure may be one cause of this; another is that the protein is weakened by attack from proteolytic enzymes, as has been demonstrated by experiments with isolated vesicles (9, 37).

Whatever the causes of variation in critical pressure are, it is clear that the blue-green algae require gas vesicles which are stronger than those of the halobacteria. Firstly, they inhabit deeper waters than the shallow brine pools occupied by the salt bacteria. But, more important, they generate cell turgor pressure of several atmospheres. This has been demonstrated, in the absence of any other suitable method for prokaryotic organisms, by using the gas vesicles themselves as devices for measuring hydrostatic pressure. The turgor pressure can be estimated from the difference

in pressure that has to be applied to collapse gas vesicles in cells suspended in a hypertonic sucrose solution, and in the normal culture medium (108). In this way turgor pressures of up to 4.5 atm have been recorded for the blue-green algae (15) and up to 1.5 atm for a purple sulfur bacterium, whereas no turgor pressure has been detected in cells of halobacteria (108), as is shown in Fig. 9. Thus it appears that the gas vesicles in these different groups of organisms are adapted to withstand the pressures to which they are likely to be subjected.

As is to be expected for a structure whose only component is a protein, its strength is affected by a range of chemical and physical factors which affect the conformations and interactions of protein molecules. The vesicles become progressively weaker in temperatures in excess of 40 C, in the presence of agents which compete for hydrogen bonds, and others (such as chloroform) which destroy hydrophobic bonding (9). Electron paramagnetic resonance studies indicate that irreversible conformational changes take place under similar sets of conditions (37). The gas vesicles of both blue-green algae and halobacteria show optimal stability at around *p*H 7.5 and demonstrate marked weakening and even spontaneous collapse at *p*H values above 10 and below 4 (8, 9). They show different behaviors in solutions of varying salt concentration, however, for while those of the alga are unaffected by concentrated solutions of monovalent cations, those of the halobacteria become considerably stronger. This obviously represents an adaptation to the salinity of the cell environment (*see above*).

Surface Properties

The inner and outer surfaces of gas vacuoles are almost certainly of a contrasting nature, for while the two opposed inner surfaces of collapsed membranes show no tendency to separate, the outer surfaces show little tendency to aggregate in aqueous suspension (89).

It is to be expected that the outer surface has a hydrophilic nature which minimizes the pressures generated on the structure by surface tension (105). With bubbles of the size of gas vesicles, surface tension would generate pressures in the order of 20 atm or more, well in excess of the observed critical pressures. Of course it could be argued that with gas vesicles, the critical pressure observed is that which the structure will stand over and above that already generated by surface tension. However, it seems that the pressure developed in this way must be very small as no significant increase in critical pressure can be detected on adding surface-active agents (9). Other observations indicate a surface readily wettable by water, and the fact that intact gas vesicles show a partition coefficient of 1.0:0.0 between water and olive oil confirms the hydrophilic nature of their outer surfaces (108).

There are reasons for thinking that the inner surface of the gas vesicle is, on the other hand, hydrophobic (89, 105), and that by this mechanism water is prevented from accumulating inside the structure. It seems likely that the gas vesicle membrane is as freely permeable to water vapor as it is to other gases. (It is possible that it might discriminate against water on account of its polar qualities, though the membranes were not found to be any less permeable to another polar gas—carbon monoxide—albeit having only one-tenth of water's dipole moment.) However, if pores in the membrane were to open into a hydrophobic surface it would require a very large pressure (equal to $\cos \theta \cdot 2\ \gamma/r$, where θ is the contact angle, γ the coefficient of surface tension, and r the pore diameter) to force *liquid* water inside the structure, and such a surface would also prevent water from accumulating inside by condensation (105).

FORMATION AND SHAPE OF GAS VESICLES

The original ideas associated with the formation of gas vacuoles were that they resulted from the metabolic production of some gas (see 22) and recently Bowen and Jensen (4), who observed the reappearance of gas vesicles in cells which had been subjected to pressure, proposed that they can reversibly collapse and expand, suggesting erection of the structure by inflation with gas. However, considering the permeability of the vacuole membranes to gases, it seems most unlikely that this could occur. I have proposed that the vesicle must, therefore, be a self-erecting structure, and suggested that it starts life as a cluster of particles, which are orientated in such a way that as more are added, a hollow structure is formed. The energy required to increase the size of the structure under the pressures impinging on it would have to be derived from the assembly process, but the gas would enter the enclosed space by passive diffusion (105). Perhaps the contrasting hydrophobic and hydrophilic surfaces presented at the inner and outer membrane surfaces, respectively, may be important in correctly aligning the particles and in the

process of their insertion into the membrane (108).

The existence of juvenile stages in gas vesicle production (105) lent some support to the above hypothesis, but the first good evidence that gas vesicles are synthesized in a de novo fashion was presented by Waaland and Branton (101). They found that gas vesicle formation in the blue-green alga *Nostoc muscorum* could be induced on dilution of its culture medium. The first gas vesicles to form were small biconical structures, which developed into short cylinders with conical ends; with time, the length of the cylinders increased but the ends remained the same. This sequence suggested to them that the new components of the membrane might be added at the middle of the cylinder as it increased in length, and they remarked that in freeze-etched material there is often a rather more prominent rib in this region, which might represent the growing point. Jost and Jones (41) say that some vesicles appear to have more than one, while others have none of these ribs, and they preferred to interpret them as stacking faults or the result of a deformation where they do occur. However, we have found that gas vesicles which collapse flat on drying onto Formvar grids often split in two about half-way along their length (111) or show a discontinuity between ribs in this region (D. Branton and A. E. Walsby, *unpublished data*; see Fig. 12c). These observations seem to indicate that each gas vesicle may be assembled in two halves. This being so, it would be expected that the two halves are identical, rather than mirror images, and this must mean that the contact relationship between the ribs at the end of each half differs from that between any two ribs within each half (Fig. 11) explaining the weaknesses observed at the junction.

Evidence has now been presented by Lehmann and Jost (61, 62) that the reappearance of gas vesicles which occurs within a few hours of collapsing existing vesicles under pressure follows a course essentially similar to that observed on inducing *Nostoc muscorum* and confirms the idea that collapsed membranes are not reinflated (105). It is still possible, of course, that the subunits of the collapsed membranes might be disassembled and reused in the construction of new vesicles. Apparently, in the first hour or so the rate at which new vesicles both form and elongate is greater than that at steady state. Lehmann and Jost speculate that this might indicate differences in the assembly processes of the conical end and cylindrical center pieces, as in the early stages of formation the juvenile forms possess only the end pieces. However, if the assembly process is normally limited by the availability of precursor subunits, then the observed increases are to be expected. This is because in the steady state the growth of a large number of existing gas vesicles has to be sustained. When these are effectively removed by pressurization, they no longer compete for the precursors, all of which may then be employed in the construction of the juvenile forms. This should be taken into account in analysis of the assembly kinetics of the different parts.

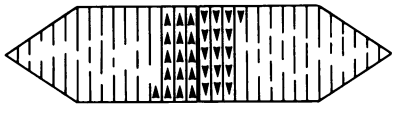

FIG. 11. *Diagram demonstrating that, if a gas vesicle is made up of two identical halves, the contact relationship between the two ribs at the center of the structure must be different from those between any two ribs within each half.*

The shape of a gas vesicle must ultimately be determined by the shape and arrangement of its constituent protein subunits. If there is but one species of protein in the algal vesicles, then the mutual relationships of the subunits must be somewhat different in the conical end caps and the cylindrical central portion of the structure; or perhaps they are quasi-equivalent in the sense used by Caspar and Klug (12) in describing polyhedral virus structures. The shape of the vesicle might then be the outcome of their being a particularly stable conformation generated by a certain number of subunits per rib, which ensures that the growing juvenile, double-coned structure changes its mode of expansion to produce a cylinder on attaining the critical size, around 70 nm. Such a mechanism might give rise to structures of highly uniform diameter, perhaps constant to the nearest subunit. In this respect, it would be worthwhile obtaining better information on the variability of this dimension in the vesicles of a given organism. It would also be nice to know if the small differences in mean diameter reported for the vesicles of different blue-green algae and certain photosynthetic bacteria (13) are genuine, and if so whether they are related to the size of the protein component. Certainly some of the estimates of vesicle diameter are in error due to distortion which occurs in negative staining (108).

The gas vesicles of the halobacteria do not have such a uniform shape. They apparently continue to grow as some approximation of a double-coned structure, indicating that no

strong restraint exists on their rib diameter. Cylindrical vesicles are occasionally encountered, but these are of variable diameter. The same appears to be true of those in the purple sulfur bacteria (see Fig. 10b) and certain heterotrophic bacteria (87, 98), though the range of size is smaller in these cases.

The end caps of cylindrical vesicles, wherever they occur, are invariably referred to as being cone-shaped (e.g., 4, 13, 41). This is the impression usually given by freeze-etching and by sectioning, but not by negative staining, where the cap usually appears to be more rounded (41) and to end in a mucronate point (see Fig. 12a) or even a short stalk (see Fig. 12b). Jost and Jones (41) find the end angle to be 70° in the vesicle of *Microcystis aeruginosa* (which gives a cone of altitude 35 nm $\times$ cot 70°, or about 50 nm), though calculations from their measurements of flattened cones suggest a substantially wider angle. Again, it would be worthwhile obtaining more detailed information on the exact shape and variability of these regions. Details of the extreme tip would be particularly welcome, as it presumably reflects the form of the vesicle in its earliest stages and may hold clues to the processes of its assembly.

FUNCTIONS OF GAS VACUOLES

Before proceeding to analyze in detail the functions that gas vacuoles might perform, we should at least entertain the possibility that they confer no advantages on the cells of the bacteria or algae which possess them. Two sorts of structure might fall under this category. The first are those which might belong to some parasite, as is the case with the viral structures discussed above. Of course, these structures are themselves essential to the virion (in protecting nucleic acid in the case of viral coat protein). The second are those structures which might be produced by the cell, but are incapable of fulfilling any useful function, or which perhaps once fulfilled a function, in an ancestral form, which has now become redundant. It is generally considered that such structures would not be retained in the face of selection pressures on evolving organisms. One is forced back to consider these possibilities seriously only if no convincing function for the structure can be found.

In setting out to assess the functions which a structure might perform, one cannot do better than to begin by quoting the four postulates of Williams and Barber (116).

(i) "The structure is necessary for successful growth of the plant (or organism) in competition with others.

(ii) The structural provision is adequate for the requirements of the function it is supposed to serve.

(iii) These requirements could not have been met with markedly greater economy by some other available means.

(iv) Provision is not markedly more than is necessary to fulfil the functional requirements."

In clearly contravening postulate (ii) the function of storing gases such as nitrogen (46) or the products of fermentation (10, 50) can be ruled out, as the membranes are far too permeable to prevent any particular gas from diffusing away (105). It seems unlikely that the extra amount of gas "contained" in a gas vacuole can be of any advantage for metabolic purposes, even in the short term, as the time for which a gas molecule is retained by the membrane is probably very small. Moreover, any gas which does enter the structure must first diffuse through the surrounding cytoplasm. It would appear, therefore, that, unless the gas vesicle membrane possesses properties other than those discussed above, any function performed by gas vacuoles must depend on the spaces they contain being kept free of solid or liquid, rather than filled with gas (105, 108).

Providing Buoyancy

Intact gas vacuoles can provide buoyancy because they have an overall density much less than that of the aqueous medium in which the various organisms grow. The probable density of the constituent gas vesicles can be calculated from estimates of the density of the membrane, its thickness, and the overall shape of the structure. Assuming the gas vesicles of blue-green algae to be cylinders of mean length 370 nm and width 71 nm capped by right cones with a solid angle of 70° (41), and the mean thickness of the membrane to be 1.62 nm (*see above*), then the ratio of membrane volume to the total volume of the structure is calculated to be 1.0:10.6. The shape of the halobacterium gas vesicles is more variable, but if we assume an oblate spheroid of length 216 nm and width 133 nm (108) then the ratio is 1.0:16.4. Then taking 1.34 g cm^{-3} as the density of the membrane (*see above*), the overall density of gas vesicle is 0.126 g cm^{-3} in the alga and 0.082 g cm^{-3} in the halobacterium. Clearly, the first two postulates are satisfied, since no solid or liquid substance is known having a density of such a low order. Indeed, if we accept that a constraint is placed on the

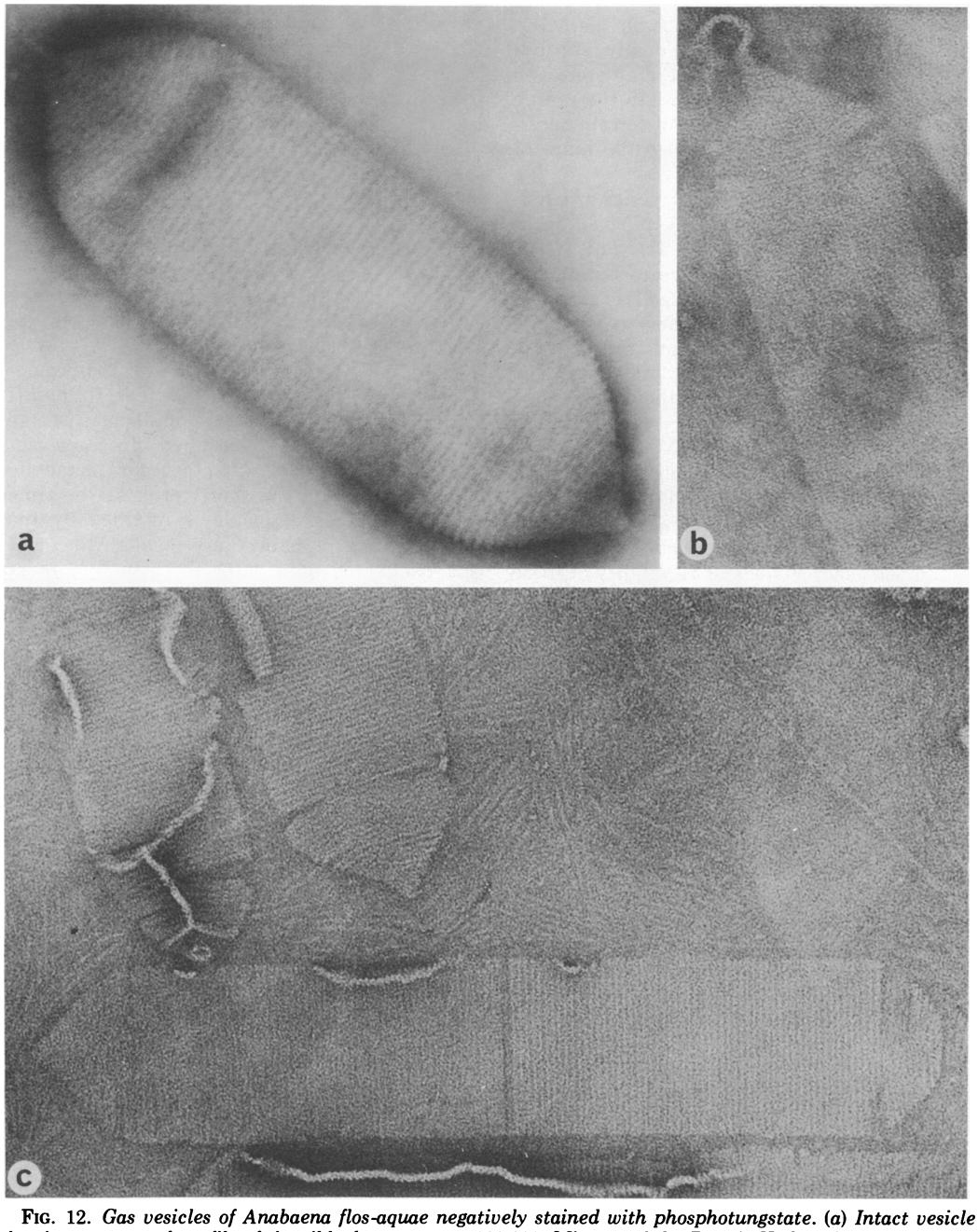

FIG. 12. *Gas vesicles of Anabaena flos-aquae negatively stained with phosphotungstate. (a) Intact vesicle showing corrugated profile of the ribbed structure, ×300,000. Micrograph by Dennis Hodge. (b) Intact vesicle showing short stalk which is occasionally encountered. Underfocussed, ×200,000. (c) Collapsed vesicles showing separation of the cone-shaped ends, and discontinuities at the central region, ×200,000. Micrograph by Daniel Branton.*

diameter a gas vesicle may reach by the pressure it must withstand, as suggested above, this explains why buoyancy is apparently provided with "more marked economy" by the halobacterium vesicles (which are subjected to less pressure) than the algal ones. In fact it can be demonstrated that the cylindrical form of the algal vesicle is the most efficient space-storing shape when rib diameter is limited by the impinging pressure (108) and that some of

the lost advantage is made good by the cylinders stacking together with less intervening space than spheroids (109).

Naturally, gas vesicles will only provide a cell with buoyancy if they occupy a sufficient proportion of the cell volume. The possession of gas vacuoles is often correlated with cells in a floating condition, not only in the alga (e.g., 22, 27, 30, 46, 75) and halobacteria (34, 57, 70) but also in the prosthecate (87) and photosynthetic bacteria (73). In almost all cases the cells can be shown to be dependent on gas vacuoles for their buoyancy, which is lost when the vacuoles are collapsed by pressure (e.g., 4, 22, 46, 50, 71, 104; but compare 7, 107). However there are some algae (14) and photosynthetic bacteria (P. Hirsch, *personal communication*) which, though possessing gas vesicles for much or all of the time, are seldom or never buoyant.

Few direct measurements have been performed on the actual volume occupied by gas vacuoles in cells. From the changes in specific gravity which occurred when the alga was subjected to pressure, Klebahn estimated that the gas-filled spaces of the vacuoles occupied 0.8% of the cell volume in *Gloeotrichia echinulata*, and that 0.7% by volume was the minimum proportion which would be required to make the alga float (46). Using the same method, I have found that with *Anabaena flos-aquae* the volume occupied by gas varies from 1.8 to 9.8%, when grown under varying culture conditions, and that about 2.1% is required to render it buoyant (107). Smith and Peat, investigating the same two algae by electron microscopy, concluded that the gas vacuoles occupy 22% of the cell volume, and in a third alga *Oscillatoria agardhii*, 39% (84). However, I understand from them that these figures include the volume occupied by the gas vesicle membranes and the spaces between them, so that comparisons with estimates of gas volume are not valid.

Petter observed that by light microscopy the cells of the halobacteria appeared to be almost filled with gas vesicles (70), and, although electron microscopy shows this view to be somewhat exaggerated (34), I have found that the vacuole gas occupies a substantially greater proportion of the cell volume than in the alga. Estimates of gas vesicle void volume, by the manometric method (46), and packed cell volume, by centrifugation, indicate a value of nearly 15% (*unpublished data*).

The excesses of buoyancy indicated by these limited studies may, at first sight, appear to contravene the fourth postulate dealing with "works of supererogation" (116). However, flotation is a time-dependent activity whose rate varies in proportion with the difference between the densities of the floating object and of the suspending medium, according to Stokes' law. For a sphere $U = 2 g r^2 (\rho - \rho')/9 \eta$, where U is the sinking rate (negative when $\rho < \rho'$), g the gravitational acceleration, r the radius of the sphere, ρ its density, ρ' the density of the medium, and η its coefficient of viscosity. Plainly, to maintain a given rate of rise, $\rho - \rho'$ must have a greater (negative) value for a small object than a large one, and this puts in perspective the higher degree of gas vacuolation in the halobacteria. In the same context, it is interesting to note that, while most other planktonic algae tend towards the unicellular habit or small, divided forms giving slow sinking rates, many gas vacuolate blue-green algae have adopted streamlined colonial forms which give high rates of flotation; viz., the flakes of *Aphanizomenon* (69), balls of *Microcystis* and *Coelosphaerium* (75), and rafts of *Anabaena*. Reynolds has shown that the observed flotation potential of blue-green algae gathered from waterblooms is correlated above all else with colony size (76, 77).

Buoyancy in the halobacteria. It has proved relatively simple to demonstrate that the putative buoyancy-providing role of gas vacuoles satisfies the last three postulates of Williams and Barber; but it is not quite so easy to decide about the first postulate, dealing with the necessity of the structure. In doing so, it is felt that affirmatives to the four questions set out below, should be sought. Although we are not yet in a position to supply all the answers, an examination of the need for buoyancy in the halobacteria seems to provide an ideal test case.

(i) *Are there reasons for thinking that the function, as and when performed by the structure, should aid the survival or growth of the organism, at least under some of the conditions to which it may be exposed?* Petter (71) has pointed out that these bacteria inhabit saturated brine pools, in which the solubility (and hence the diffusive transfer rate) of oxygen is very low, and gas vacuoles enable the bacteria, which are obligate aerobes, to float to the surfaces of the pools, thus ensuring their access to air (57).

(ii) *If the structure is removed from the organism, or rendered ineffective, does it have detrimental effects on the growth or survival of the organism?* This might be difficult to test with some structures but it is extremely simple with the gas vacuole, which may be col-

lapsed by pressure. It would be interesting to compare the growth rates of the vacuolate and nonvacuolate halobacteria in static cultures (where buoyancy should provide advantage) and shaken cultures (where no advantage would be conferred).

(iii) *If a stable mutant lacking the structure occurs, is it selected against under conditions which permit the execution of the function?* Both *Halobacterium halobium* (89) and *Halobacterium* sp. strain 5 (57) form mutants which lack gas vacuoles. In the latter, this mutant arises quite frequently, and no back mutation is observed (unless induced by mutagens, see reference 89). Larsen et al. (57) reported that when this strain is repeatedly subcultured on agar, the proportion of the gas-vacuolate mutant progressively increases. Buckland has found that when serial subcultures are made from static liquid cultures, there was no such increase (8). However, we are reluctant to conclude, at this stage, that this demonstrates the selection of the gas-vacuolate form due to its buoyancy, since the increase has not been observed in shaken cultures, where buoyancy should be of no consequence.

(iv) *If the structure is an inducible character, do the conditions which promote its formation correlate in any way the need for the function it performs?* It is not clear whether gas vacuoles can be regarded as inducible systems in the halobacteria, but it has been observed that they are most abundant in the late exponential and stationary phases of growth and absent, or of low frequency, during the early exponential phase (8, 57, 89). One condition that might be correlated (negatively) with this sequence of events is oxygen tension which will fall, even in shaken cultures as the population density rises. There are indications that high oxygen tensions inhibit gas vacuole formation (8), but again the evidence is, as yet, inconclusive.

In other heterotrophic bacteria. As yet we have very little information on the physiology of the other heterotrophic bacteria listed in Table 1, but it is clear that in certain of the forms which have been observed in culture (87, 97) that the gas vacuoles render the cells buoyant such that they float at the water surface. For the aerobic organisms, buoyancy is likely to have the same significance as that proposed for the halobacteria, increasing the availability of oxygen. Not all of the heterotrophic forms described are aerobes, however. The possibility and significance of gas vacuoles being used to *regulate* buoyancy in the anaerobic forms is discussed, briefly, below.

In blue-green algae. Paradoxically, several of the early reports of gas-vacuolate algae were of forms occupying the sapropel or bottom muds (14, 22). It was suggested that the gas vacuoles might form specifically under the anaerobic conditions prevailing in these habitats, causing the cells or filaments to float to the water surface (50). It has also been claimed that gas vacuole production is induced in some algae by a change in the concentration of mineral salts in the culture medium (10, 101) or a change in light intensity (14, 63, 95, 103). However, in at least certain of these cases gas vacuole production is restricted to particular types of cell whose differentiation may be the primary event triggered by changing conditions. For example, Canabaeus (10) reported that, in both *Tolypothrix rivularia* and *Calothrix epiphytica*, gas vacuole formation was restricted to the hormogonia into which the tapering ends of the filaments differentiated, and similar observations have recently been made with *Gloeotrichia ghosei* (81). The hormogonia are buoyant when they are released and this may be important in the dispersal of species having sedentary stages.

The majority of truly planktonic blue-green algae possess gas vacuoles at all times and may aggregate at the surfaces of natural waters where they form waterblooms. Reynolds, who investigated the formation of several such blooms on meres of the Shropshire-Cheshire Plain, in England, reported that alga taken from the lake surfaces always possessed gas vacuoles, and owed its buoyancy to them (76). By taking depth profiles of algal concentration in the water, he demonstrated that the sudden appearance of a bloom did not result from an explosive increase in algal numbers, but was a consequence of the "telescoping" of algae growing throughout the water column to the lake surface (75). This only happened in calm weather when turbulence, which tended to mix the alga back down into the water mass, subsided (64, 75).

Superficially, it might seem that the conditions existing at a lake surface would be those which were most suitable for algal growth. It has been suggested that carbon dioxide is limiting in some waters (54) and that this might be more available to algae floating in contact with air at the surface (107). The mean light intensity, too, is higher at the top of the water column, and should sustain higher rates of photosynthesis. However, surface light intensities may, of course, become high enough to inhibit growth, and even cause death (*see be-*

low) though the sensitivity of different species seems to vary considerably in this respect. For example, studies in progress on Clear Lake, Northern California, have revealed that while *Aphanizomenon flos-aquae* may not survive for more than a few hours at the lake surface, *Microcystis aeruginosa* apparently thrives in this situation (A. J. Horne, *personal communication*). Another factor which may lead to the demise of the algal population at the water surface is the exhaustion of nutrients by the concentrated algal mass (24). When light winds fan the surface of the water without actually inducing turbulence in it, the alga may be swept to the leeward shore, and produce a thick surface scum (77). Once subjected to these conditions, the alga rarely remains viable (20, 25, 75; but contrast 107). Probably, for many species, the appearance of a waterbloom marks the end of an algal population rather than its climax, though as with most good shows the climax may come very close to the finale.

It thus seems likely that the buoyancy provided by gas vacuoles has some more subtle significance to many planktonic blue-green algae than merely that of carrying them to the water surface. The same must be true of gas vacuoles in the photosynthetic bacteria, since these organisms are obligate anaerobes which would not survive exposure to light in the aerated medium of water surfaces (72). Lund (64) has pointed out that the buoyancy provided by gas vacuoles will produce "forced convection" as the alga (or bacterium) moves with respect to the water mass, and that as with sedimenting algae this may be of importance in maintaining around the cells steep diffusion gradients of nutrients (68).

Lund also remarked on the paradox of algae with gas vacuoles maintaining a position someway below the lake surface. That this even occurred in the waters under ice, in which turbulence is completely absent, demonstrated that the alga could not be lighter than water (64) and provided the first suggestion that gas vacuoles might be important in regulating buoyancy.

Regulating Buoyancy

Variation in the ratio of cell volume to gas-vacuole volume may be brought about by changes in the respective rates at which cell materials and *intact* gas-vacuoles (i) are produced, and (ii) disappear. Such changes will, in theory, result in variation of the cell density and will determine whether the cell sinks or floats, and how rapidly it does so. If the changes are correlated with physical or chemical conditions which form vertical gradients in natural waters, they may provide the cells with a means of regulating their position in the vertical water column. There are a number of conditions which may vary with depth, and, in particular, light intensity, temperature, salinity, oxygen tension and pressure may form stable gradients.

In blue-green algae. The degree of gas vacuolation in various blue-green algae has been reported to respond in different ways (contrast reference 105 with 103) to light intensity, but in three planktonic forms which have been investigated, *Anabaena flos-aquae* (15, 105, 108), *Oscillatoria redekei* (114), and *Microcystis aeruginosa* (15), gas vacuoles are recorded as being more abundant in alga grown under low light. Two mechanisms have been proposed to explain this.

The first is that the formation of gas vacuoles may proceed independently of light intensity, with the result that as this increases they become diluted out by the greater vegetative growth that this sustains (107). Smith and Peat (85) found that gas-vacuolation of *Anabaena flos-aquae* was least in the early exponential phase when growth of the alga was most rapid; and Lehmann and Jost (61) demonstrated a similar response in *Microcystis aeruginosa* by the gas vesicle-counting method (42). They concluded that the decrease in gas vesicle numbers must have resulted from dilution by cell growth, regarding the disappearance of these structures as being unlikely because of their "high stability." It has been pointed out, on the other hand (105), that the fall in gas-vacuolation may be a direct response (perhaps by the second mechanism) of the cells to the higher light intensities which exist in young cultures.

The second mechanism is that the alga disposes of intact gas vesicles in high light intensity by collapsing them (108). It has been found that when *Anabaena flos-aquae* is grown under low light intensity its cells have a lower turgor pressure and a proportion of the gas vesicles with a lower critical pressure than when the alga is grown under high light (108). When the alga was transferred from low to high light intensity, the cell turgor pressure was found to increase in instances from less than 3 to more than 4.5 atm (290 to 460 kN m^{-2}) and at a rate of nearly 1 atm (94 kN m^{-2}) per hr. The rise in turgor pressure, which results from the increased accumulation of photosynthate, was

found to be sufficient to collapse enough of the alga's gas vesicles to destroy its buoyancy. The loss of buoyancy was often quite rapid, being detectable within half an hour, and nearly complete at two hours (15). Sometimes rather more gas vesicles appeared to be lost than could be accounted for by the rise in turgor alone and while this may have been an artifact of measurement (15) it is also possible that some vesicles collapsed as a result of their becoming weakened in a changing cellular environment. We have found that several factors affect the strength and stability of isolated gas vesicles, and two of these, decrease in *p*H (as might occur with the production of organic acids) and exposure to proteolytic enzymes, might be important in weakening them inside cells (9). This possibility requires further investigation.

It is plain that any response which results in decreased gas-vacuolation with increasing light intensity could enable the alga to select a position on a vertical light gradient (108), and there are several accounts of blue-green algae doing this in freshwater lakes (18, 19, 64, 119; J. W. G. Lund, quoted in reference 26). Fogg (25) has argued that the conditions which exist in the discrete layers occupied by these algae may be those most suitable for their growth. In fact it has been observed that as inorganic nutrients become depleted in certain freshwater lakes, the population maxima of gas-vacuolate blue-green algae (but not other forms) move down (18, 119). This suggests that the algae station themselves at a depth where the mean light intensity does not exceed that required to maintain growth at a level which is limited by other factors (26). It has been proposed that just such a response would be provided by the mechanism described above in which the rise in turgor pressure results from the amount of carbon fixed in photosynthesis exceeding that which can be assimilated by the cells (15).

The collapse of gas vacuoles under rising turgor pressure is sufficiently rapid to explain another curious behavior pattern of blue-green algae, that of diurnal migration to and from the water surface during night and day, respectively (30, 82). This behavior would also seem to be connected with avoiding high incident light intensities, particularly in the case of the tropical lake described by Ganf (30).

In photosynthetic bacteria. It has been suggested that the sensitivity of blue-green algae to high light intensities, particularly in the presence of oxygen (26), bears the mark of their affinities with photosynthetic bacteria (107). These organisms show a requirement for that strange combination of light and anaerobic conditions provided only below layers of water, which although transparent to light provides a diffusion barrier to oxygen. Thus, whereas recent observations concerning the great suitability of layers beneath the surfaces of fresh-water lakes for blue-green algal growth (25) may be met with some surprise, parallel observations form part of the well established lore of the photosynthetic bacteria. Pfennig (72) has recently reviewed the literature documenting cases of purple and green photosynthetic sulfur bacteria occupying the anaerobic zones at or below the thermoclines of various lakes, down to depths as great as 35 meters. Many of the bacteria concerned are motile "and able to migrate by their own flagellar activity," but others (e.g., *Rhodothece*, *Thiopedia*, *Lamprocystis*, and *Pelodictyon*) although immotile, possess gas vacuoles, and "will rise passively as long as they are less dense than water, which is mostly true for water at 4 to 8 C." This statement suggests a simple mechanism whereby, in possessing gas vacuoles, cells achieve a minimum density that is less than that of cold water beneath the thermocline but greater than that of the warmer water above it. However, subsequent investigations by Pfennig and Cohen-Bazire (73), who found that cells of *Pelodictyon clathratiforme* formed gas vacuoles and became buoyant when grown in dim light at between 4 and 8 C, but not at higher temperatures, indicate a more complex mechanism. This response may be regarded as parallel to that of the blue-green algae and indicates a similar buoyancy-regulating mechanism. Studies with the purple sulfur bacterium *Rhodothece conspicua* [= *Amoebobacter roseum* (*Bergey's Manual*, 8th ed., *in preparation*)] have shown that the capacity to collapse gas vesicles under cell turgor pressure exists in these organisms (108) and it is possible that a rise in turgor may be promoted by high light intensity and elevated temperatures. Some of these organisms occur abundantly at the more permanent thermocline in meromictic lakes (13, 72), and it is tempting to speculate on the outcome of a possible turgor pressure rise resulting from the cells floating from the saline to the overlying freshwater layers in this region.

In heterotrophic bacteria. Van Ert and Staley (98) have recently isolated a gas-vacuolate, heterotrophic bacterium (possibly a species of *Achromobacter*) which is a facultative anaerobe. They reported that gas vacuole production by this organism is sporadic but seems to be favored when the cells are grown

in liquid culture media under reduced oxygen tension. Perhaps this organism, which was collected from the oxygen-depleted thermocline of a eutrophic lake, possesses a buoyancy-regulating mechanism which responds to the concentration of oxygen in the water. It would also be interesting to know if such a response existed in *Sarcina ventriculi* (50), which is an *obligate* anaerobe (33).

Hirsch and Pankratz (32) have described other gas-vacuolate bacteria, some of which may be heterotrophic organisms, occurring in the *Thiopedia* layer on the mud surface of a shallow pond. Presumably these cells either are anchored to a substrate which prevents them from rising (they were selected by their ability to attach to Formvar-coated grids immersed in the pond) or have insufficient gas vesicles to render them buoyant. The role of gas vacuoles in these forms is not immediately apparent. It may be that these organisms also stratify at the anaerobic thermoclines of deeper waters in which situation buoyancy regulation is important, and do not have the capacity to lose their gas vacuoles completely under the equivalent conditions offered in the sapropel of shallow pools.

Light Shielding

The idea that gas vacuoles might perform a light shielding function was first suggested by Lemmermann (63), on noticing that they were invariably present in the algae of surface waterblooms, which might be exposed to high, perhaps damaging, light intensities. This concept has been supported by the observation that optical changes accompany the destruction of gas vacuoles by pressure, and perhaps also by the reddish appearance of the structures under the light microscope.

Now that gas vesicles have been isolated and purified, it is plain from the results of chemical analysis, and the white appearance of intact gas vesicles in suspension, (38, 89, 111) that they are free from pigments. Thus, their reddish hue must be a structural color (118), and any shielding they perform must be a result of structural interaction with light. Four types of interaction have to be considered: refraction, reflection, interference, and scattering. Apart from interference, which can result in the annihilation of light rays, these structural interactions will result only in the redirection of light through a suspension of cells, and not a diminution of its intensity. However, the gas vesicles may be so located and oriented in the cells that they direct light away from underlying light-sensitive structures. Refraction was suggested to be the cause of the red appearance of gas-vacuoles by Petter (71). She argued that red light would be bent less than blue on passing across the air-water interfaces of the vacuole and consequently, at certain levels of focus, more red light would be collected by the objective lens. In this way, gas vacuoles might provide a measure of protection from light of shorter wavelength when they are interposed in the path of incident light rays. Analysis of gas vacuoles under the interference microscope by Fuhs (28, 29) shows their overall refractive index to be close to 1.1, the value expected considering the combination of their gaseous contents (which would have a refractive index of close to 1.0) and membranous substructure.

Internal reflection provides another way in which light might be redirected back out of the cell, as proposed by van Baalen and Brown (96). The same authors propose that there might also be "an interference effect perpendicular to the long axis" and that, for gas vesicles of average diameter 78 nm, "zero order reflection will occur [at a wavelength] around 320 nm." No explanation is tendered. As with reflection, the angle the incident light ray makes with the gas vesicle will be extremely important. The strongest interference effects are created by planar surfaces (rather than cylindrical ones). I have observed that thin films of gas vesicles spread on water, like oil films, produce rainbow effects, indicative of interference, but there is no evidence that this effect can produce light shielding of any value inside cells, even considering the particular configurations which gas vesicles adopt.

On account of their minute size and the large difference in refractive index between their gaseous contents and the aqueous suspending medium, gas vesicles produce very strong light scattering (37, 106, 107). Over most of the visible range, the intensity of the light scattered shows the relationship $i \propto \lambda^{-4}$ characteristic of Tyndall or Rayleigh scattering (106, 107). Gas vacuoles in cells show basically similar characteristics, though because of the presence of other scattering and absorbing material, and also for reasons discussed below, it is less easy to analyze. There seems little doubt that gas vesicles singly or stacked together can scatter light away from underlying objects, though just what proportion of the incident irradiation of any given wavelength a single layer of gas vesicles will scatter has yet to be determined. One feature of interest, however, is that the shorter wavelengths, which are the most damaging, will be scattered away much

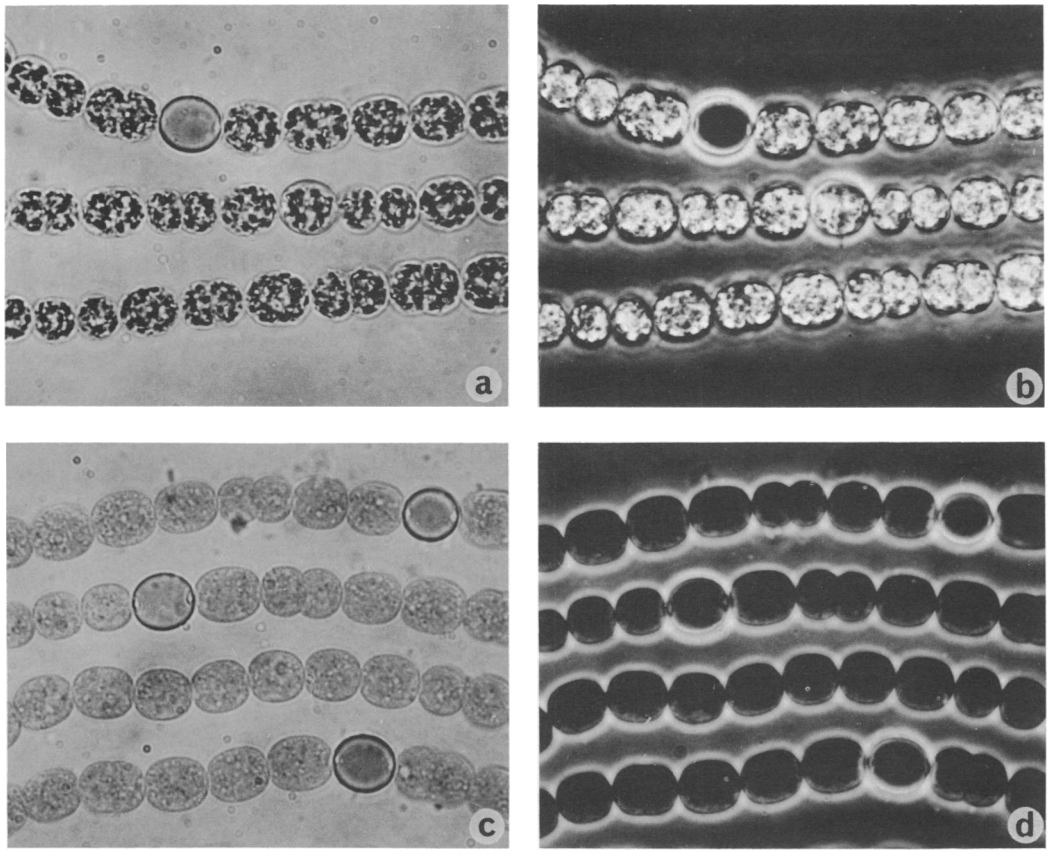

FIG. 13. *Light micrographs of filaments of Anabaena flos-aquae (a, b) with gas vacuoles; (c, d) filaments from the same sample whose gas vacuoles have been collapsed under pressure. (a, c) Bright field; (b, d) positive phase-contrast illumination. (The clear cells are heterocysts.) Fuhs (28) points out that gas vacuoles are excellent phase objects because they have a refractive index much lower than the surrounding cytoplasm. When the difference in optical path length exceeds the retardation of the phase plate, this results in false reversal, and they appear brighter than the background (as in b). They do not absorb light and thus are poor amplitude objects, almost invisible under bright field with high resolution optics; but with low numerical apertures they are characterized by diffraction fringes (as in a).*

more strongly than the longer ones (which are perhaps more useful in photosynthesis). This differential scattering effect also probably contributes to the red appearance of gas vacuoles viewed by transmitted light.

Plainly, the theoretical aspects of the ways in which gas vesicles interact with light have received but scant attention to date. It is felt that, of the effects considered, light scattering is the most important, but that a much more detailed analysis of the problem is required.

In each of the above considerations it is emphasized that the gas vacuoles should be orientated between the incident light and the light-sensitive structure. The latter comes in two categories: the photosynthetic apparatus whose pigments may catalyze or be susceptible to photooxidations (93), and proteins and nucleic acids which can be damaged by ultraviolet radiation (35). Gas vesicles are usually located in the center of the cell or throughout the cytoplasm in most blue-green algae (22) and bacteria (13). However, there are accounts of three blue-green algae which, at least under certain conditions, have gas vacuoles distributed at the periphery of their cells. The first is *Trichodesmium erythraeum*, a marine alga which, floating at the surfaces of tropical seas, is exposed to very high illumination. Its vacuoles form a hollow tube running nearly the entire length of each cell surrounding most, but not all, of the photosynthetic lamellae (96).

The second is a strain of *Nostoc muscorum* in which gas vesicle formation is induced only under certain specific conditions, viz., on dilution of the culture medium, when they form throughout the cell (101), and on transfer to a higher light intensity (>4500 lx), when they formed parietally in the cell (102). The third is *Anabaena flos-aquae*, in which the gas vacuoles, while showing a quantitative decrease when the alga is transferred to a higher light intensity (15, 105), do exhibit a different distribution, forming at the cell periphery, rather than throughout the cytoplasm (H. Shear and A. E. Walsby, *manuscript in preparation*). Taken with the theoretical considerations outlined above, these observations appear to support the idea that gas vesicles provide a light shielding function; recently two empirical approaches have been made in an attempt to see if they do.

In the first, the Waalands and Branton (103) examined the absorption spectra of *Nostoc muscorum* using a spectrophotometer which corrected for a proportion of the light scattering by samples (i.e., the photocell collected much of the scattered as well as the transmitted light). When isolated vesicles were added to a suspension of nonvacuolate alga, the difference between the absorption spectra with the vesicles intact and with them collapsed was a smooth curve, nearly the same as that given by isolated vesicles in the absence of the alga. However, the difference between the spectra of alga with intact and with collapsed vesicles *in the cells* showed distinct troughs at the position of the major pigment absorption peaks (in the blue and red). They construed this as demonstrating that when the gas vesicles were present in the cells (but not in suspension outside of them) they were scattering light away from the cells in such a way that it was not available for absorption by the underlying structures, and in this manner they shielded the photosynthetic apparatus. The fact that, on collapse of the gas vacuoles, one of the absorption peaks actually *increased* a little, though only by 3%, does demonstrate that there must have been some shielding. However, the occurrence of the troughs in the difference curve must be due *in part* to an artifact of measurement (Shear and Walsby, *in preparation*). Duysens (17) has shown that less light is absorbed by a sample when the pigment it contains is present in suspended particles than when the pigment is homogeneously distributed in solution, and that this effect is greatest at the absorption peaks. Consequently, on removal of the pigment from the particles, the decrease in absorption at the peak wavelengths is less than would be expected from measurements on the extracted pigment. We have shown that a comparable situation arises at the absorption maxima on removal of the light-scattering vacuoles (again, using a spectrophotometer which is not *totally* corrected for light scattering) and occurs irrespective of whether the gas vesicles are present in the center or periphery of the cells (Shear and Walsby, *in preparation*). Thus, in order to assess the degree of shielding by spectrophotometry it would be necessary first to estimate the contribution of this effect by applying the theoretical corrections described by Duysens (17).

Shear and I have attempted to estimate the degree of shielding directly by comparing the rates of various photochemical events in cells of *Anabaena flos-aquae* with and without intact gas vacuoles. We investigated photosynthesis over a range of limiting light intensities; the photooxidation of phycocyanin (which did not show photochemical equivalence) under intense illumination; and the survival of the alga after exposure to ultraviolet light (which should be scattered very strongly). In no case were significant differences recorded between algae with gas vacuoles, and those without, either with low-light-adapted (with central gas vacuoles) or with high-light-adapted alga (with peripheral vacuoles). There were differences between the two sets of alga, though these must have been due to some other cause, presumably pigmentation (*manuscript in preparation*). There is thus no evidence that gas vacuoles provide a significant degree of shielding in this alga. The different orientation of the gas vacuoles under high-intensity light remains a mystery at present but holds out a warning to the dangers of jumping to conclusions from teleological evidence.

It is suggested that it would be a simple matter to test the effectiveness of gas vacuoles in other algae (especially the *Nostoc muscorum*) and halobacteria (which inhabiting the surfaces of brine pools are also subjected to high and perhaps damaging light intensities) by measuring the resistance of these organisms to ultraviolet light. Pending these results it is wondered how the light-shielding function will stand in relation to the third postulate of Williams and Barber (116). Carotenoids, which apparently fulfil this function (53), occur abundantly in blue-green algae (23), photosynthetic bacteria (72) and halobacteria (16). However, in comparing the economy of gas vacuoles and carotenoids in providing light shielding, it

should be remembered that if gas vacuoles are already fulfilling another function, such as providing buoyancy, then the *additional* expense involved in their providing light shielding (say by reorientating the structures) may be small, representing an overall saving even if they shield relatively inefficiently. Moreover, if they are effective, they should be particularly so in the ultraviolet wavelengths where carotenoids offer no protection (55).

Light shielding in suspensions en masse. Even if gas vacuoles do not provide shading directly for other structures within a given cell, their presence may significantly affect the passage of light through a suspension of such cells. If the suspension is illuminated from above, as in direct sunlight, then back-scattering of light from the superficial layers will serve to decrease the amount of light entering the suspension. On the other hand, scattering might also increase the effective path length of light through the suspension, and so increase the effective absorption within a given layer. This in turn will result in the mean light intensity falling off more rapidly with depth, so that cells with gas vacuoles in surface layers might be considered as providing sacrificial shading for cells in the layers below them.

Increasing Cell Surface Area to Volume Ratio

Houwink has pointed out that the presence of gas vacuoles will result in an increase in the ratio of cell wall area to cytoplasm volume, though he questioned whether this would be profitable to the organism (34). It would, theoretically, bring about an increase in the rate of exchange of substances by the cell, and it might also allow the accommodation of a greater quantity of a metabolically active surface membrane.

There are, or course, other ways of increasing the surface area to volume ratio (by convoluting the surface, or decreasing cell size) and while this does not prove that gas vacuoles are unimportant in this respect, it is emphasized that their capacity for doing this is very limited. Assuming a spherical cell geometry is retained, introducing gas vacuoles to 15% of the cell volume (the largest proportion observed, *see above*) would increase the surface area by only about 10%.

CONCLUDING REMARKS

Gas vesicles are clearly most unusual structures without parallel in the biological or physical world. They have, at the same time a rare simplicity which deserves thorough investigation and promises the reward of perhaps a more complete understanding than is likely to be obtained with any other subcellular organelle in the near future.

The fact that gas vesicles retain their unique properties on isolation and purification not only facilitates such investigations but also suggests that they may be put to work in spheres well removed from biology. Inside cells, their usefulness in measuring cell turgor pressure has been demonstrated (15) and they might be employed in the measurement or detection of pressures generated in abiotic systems. It has been suggested that they could be used to measure accelerations, decelerations, and the hardness of materials, from the observation that their collapse can detect pressures developed momentarily in collisions (108). Their extremely high light-scattering properties may also have application in optical fields.

To date, the structure and functions of gas vacuoles have been little investigated in the various bacterial groups, compared with those in the algae, and parallel studies are clearly required. The prokaryotic groups in which gas vacuoles have been discovered are widely separated on the phylogenetic tree and it may well be that they are of even more widespread occurrence. Aquatic habitats, including the sea, from which no gas-vacuolate forms have yet been reported, would seem to be the best place to look (13). Van Ert and Staley (98) have expressed the view that gas-vacuolate bacteria are common inhabitants of lake waters and attribute the fact that so few have been isolated to the inability of these bacteria to form gas vacuoles or even to grow in "typical" laboratory media. It is also possible that gas-vacuolate forms have been overlooked in the scrutiny of water samples by the standard sedimentation procedures, either because the cells float or because their gas vacuoles are destroyed on addition of preservatives. Methods of collection (such as sealing without an air space in screw capped bottles) and concentration (by filtration or centrifugation) which can result in the generation of pressure on samples should also be avoided. Considering the recently popularized theory of the prokaryotic origin of chloroplasts and mitochondria (11, 79), perhaps we might apply the same precautions in looking for gas vacuoles in these organelles of eukaryotic organisms (113). However, it is doubted that they could ever occupy a sufficient proportion of the total cell volume in these cells to provide useful buoyancy, and this may have militated against their selection.

Finally, it is suggested that the information we have gathered on the stability of gas vesicles under various conditions (9) might also be employed in their destruction. If gas vacuoles are so important to the success of planktonic blue-green algae which form waterblooms, we might be able to control these nuisance organisms by collapsing their vacuoles. Pressures generated by explosions have been found effective in this respect (104), and field trials are in progress (107); but it is hoped that fundamental studies on these curious structures might lead to less catastrophic solutions.

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